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Investigation of novel biomarkers and treatment strategies in steroid-resistant nephrotic syndrome

Investigation of novel biomarkers and treatment strategies in steroid-resistant nephrotic syndrome

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Abstract

Steroid-resistant nephrotic syndrome (SRNS) is a heterogeneous condition with significant numbers of patients progressing to end-stage renal failure (ESRF) requiring transplantation. After transplant, around half suffer disease recurrence, suggesting the existence of circulating pathogenic factors. The aim of this study was to investigate the interplay of phenotype, genetics and potential biomarkers with response to treatment and long-term outcome.

Detailed phenotyping of patients with SRNS, all of whom had had genetic testing, was used to examine for associations between baseline characteristics and outcomes. Novel biomarkers were sought in plasma by using mass spectrometry-based proteomics. The effects of putative circulating factors were investigated by treating podocytes *in vitro* with plasma samples followed by phosphoproteomic analysis of cell extracts.

This study found a genetic cause in 21.2% of patients with SRNS who underwent clinical genetic testing (n = 255) and in 27.8% who had whole exome sequencing (n = 187). Those with genetic disease were significantly more likely to progress to ESRF but none suffered post-transplant recurrence. Patients with secondary steroid resistance were highly unlikely to have a genetic cause and more frequently suffered recurrence. Only approximately 25% of patients had a complete response to the first intensified immunosuppressive (IIS) agent. Any response to first IIS was associated with a highly significantly lower risk of progression to ESRF compared with no response. Plasma proteomics identified uteroglobin as a potential biomarker, being raised at the time of relapse versus remission, although further validation is necessary. Phosphoproteomics suggested increased phosphorylation of palladin in podocytes after treatment with relapse plasma. Given the role of palladin in actin cytoskeleton remodelling, this protein is worthy of further investigation.

Stratification of patients with SRNS by genetics, pattern of steroid resistance and response to first IIS should be considered in future clinical trials and may help with targeting treatments.

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Firstly, I would like to thank my supervisors Dr Gavin Welsh and Professor Moin Saleem for giving me the opportunity to undertake this PhD and for their ongoing support and encouragement. Gavin has always been on hand to advise about experimental design and analysis especially in the early stages on my return to laboratory research. Moin has provided the clinical insight and helped me to appreciate the translational aspects of my research.

I am grateful to all members of Bristol Renal especially Drs Carl May, Dan Henson, Abigail Lay, Jenny Hurcombe, Ruth Rollason, Yasuko Kobayashi and Mrs Lan Ni for teaching me laboratory techniques; Dr Agnieszka Bierzynska for helping me to understand whole exome sequencing and genetic variant analysis; Mark Graham for helpful discussions about proteomic analysis; and Drs Liz Colby and Maryam Afzal for their work managing the RaDaR SRNS and NephroS studies.

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Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is as follows.

Chapter 2: Whole exome sequencing analysis was undertaken by Dr Agnieszka Bierzynska of Bristol Renal. An earlier version of the data has been previously published in our co-authored article in Kidney International (Bierzynska *et al*. 2017) [1]. Subsequently, further follow-up data were collected and an additional 3 patients identified as having genetic disease. The clinical data collection, analysis and all figures are the work of the author.

Chapter 4: All genetic sequencing and analysis were undertaken by staff at Bristol Genetics Laboratory. The text and data of this chapter were previously published in the Journal of Medical Genetics (Sen *et al.* 2017) [2]. Figure 4.3 showing *NPHS1* and *NPHS2* copy number variants is the work of Geoff Woodward at Bristol Genetics Laboratory.

Chapters 5 & 7: TMT labelling of proteomic samples, phosphopeptide enrichment, LC-mass spectrometry and initial data processing were performed by Dr Kate Heesom and staff at the University of Bristol Proteomics Facility. Proteomic data relating to five patients with steroid-sensitive nephrotic syndrome were provided by Dr Yasuko Kobayashi. All data analysis was conducted by the author.

Any views expressed in the dissertation are those of the author.

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List of Abbreviations

ABB	Albumin binding buffer
ACEi	Angiotensin converting enzyme inhibitor
ACR	Albumin:creatinine ratio
AHSG	Alpha-2-HS-glycoprotein, also known as Fetuin A
APC	Antigen-presenting cell
ARB	Angiotensin receptor blocker
AS	Alport syndrome
BGL	Bristol Genetics Laboratory
CGT	Clinical genetic testing
ci-hPod	Conditionally-immortalised human podocyte
CKD	Chronic kidney disease
CNI	Calcineurin inhibitor
CNS	Congenital nephrotic syndrome
CNV	Copy number variant
CR	Complete remission/response
DDS	Denys-Drash syndrome
DMS	Diffuse mesangial sclerosis
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ESL	Endothelial surface layer
ESRF	End-stage renal failure
EVP	NHLBI Exome Sequencing Project
ExAC	Exome Aggregation Consortium
FAC	Focal adhesion complex
FAK	Focal adhesion kinase
FDR	False discovery rate
FP	Foot process

FR-SSNS	Frequently-relapsing steroid-sensitive nephrotic syndrome
FS	Frasier syndrome
FSGS	Focal segmental glomerulosclerosis
GAG	Glycosaminoglycan
GBM	Glomerular basement membrane
GEnCs	Glomerular endothelial cells
GFB	Glomerular filatration barrier
GFR	Glomerular filtration rate
HUS	Haemolytic uraemic syndrome
IgG	Immunoglobulin G
IIS	Intensified immunosuppressive
INS	Idiopathic nephrotic syndrome
IQR	Interquartile range
IV	Intravenous
KDIGO	Kidney Disease Improving Global Outcomes
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LDL	Low density lipoprotein
LM	Light microscopy
LP	Likely-pathogenic
LPS	Lipopolysaccharide
MCD	Minimal change disease
MesPGN	Mesangioproliferative glomerulonephritis
MHc	Mesangial hypercellularity
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
MN	Membranous nephropathy
MPGN	Membranoproliferative glomerulonephritis
MW	Molecular weight
NephroS	National Study of Nephrotic Syndrome
NFAT	Nuclear factor of activated T cells

NGAL	Neutrophil gelatinase-associated lipocalin
NGPPSR	Non-genetic presumed/primary steroid resistance
NGS	Next-generation sequencing
NHS	National Health Service
NS	Nephrotic syndrome
PAR	Protease activated receptor
PEx	Plasma exchange
PR	Partial remission/response
PSR	Primary steroid resistance
RaDaR	National Registry of Rare Kidney Diseases
RCT	Randomised controlled trial
RRT	Renal replacement therapy
SD	Slit diaphragm
SDNS	Steroid-dependent nephrotic syndrome
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
SNV	Single nucleotide variant
SR	Steroid-resistance
SRNS	Steroid-resistant nephrotic syndrome
SSNS	Steroid-sensitive nephrotic syndrome
SSR	Secondary steroid resistance
suPAR	Soluble urokinase plasminogen activator receptor
TBMN	Thin basement membrane nephropathy
TNF	Tumour necrosis factor
uPCR	Urine protein:creatinine ratio
VASP	Vasodilator stimulated phosphoprotein
VUS	Variant of unknown significance
WES	Whole exome sequencing
WGS	Whole genome sequencing

Chapter 1 Introduction

Nephrotic syndrome (NS) is a rare condition, particularly affecting children, characterised by swelling of the face and other parts of the body due to loss of protein in the urine. Around 80-90% of patients respond initially to the first-line treatment which is steroids. For those who are steroid-resistant, there is a significant risk of progression to end-stage renal failure needing dialysis and transplantation. Unfortunately, around half of the patients who are transplanted suffer recurrence of the disease. Currently, our ability to predict the disease course for an individual patient, and therefore intervene with the most appropriate treatments to prevent progression, is limited. It is clear that patients with NS are a heterogeneous group. Research, particularly over the last decade, has helped to stratify patients into more similar subgroups which hopefully share a similar disease biology and thus open the door for more targeted therapies. This study uses data and samples from cohorts of patients with steroid-resistant nephrotic syndrome (SRNS) to explore the associations between clinical presentations, genetics, potential circulating biological markers and outcomes.

The Introduction will first explain the structure and function of the kidney with particular focus on the key cell affected in NS: the podocyte. Since the identification of the first genetic cause of NS in 1998, mutations in over 50 genes have been identified to be responsible for approximately 30% of cases of SRNS in childhood. Many of these genes have a role in podocyte structure or function. In the remaining 70% of patients, a "circulating factor", possibly immune-mediated, has been hypothesised. The evidence for this and some of the proposed candidates are discussed below. Subsequent sections review the current disease-

modifying treatments in SRNS, many of which have been used for decades. Some small studies of novel biologic treatments have been reported but results have generally been disappointing. One explanation may be our incomplete understanding of the pathogenesis of SRNS and therefore inclusion of patients in studies with diverse biologies. The way forward may be detailed phenotyping and genotyping of patients together with development of biomarkers which can be used together for stratification. The use of relatively recent technologies, in particular whole exome sequencing and proteomics analysis for biomarker discovery in relation to SRNS will be explored.

1.1 The Kidneys: Structure and Function

The kidneys in humans are a pair of organs situated posterior to the abdominal cavity which function to filter and remove waste substances from the blood; maintain water, electrolyte and acid-base balance; control blood pressure and process hormones including vitamin D and erythropoietin [3]. Each kidney consists of approximately 1 million functioning units called nephrons. Filtration occurs in the glomerulus with small molecules passing from the plasma into the Bowman's capsule (Figure 1.1). Reabsorption of most of the water and electrolytes occurs in the proximal convoluted tubule, loop of Henle and distal convoluted tubule.

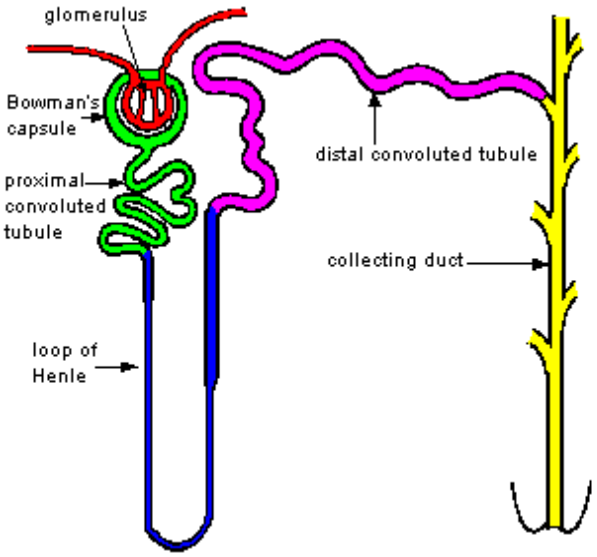


Figure 1.1: Schematic diagram of a nephron

Adapted from: http://biologymayhem.blogspot.com/2011/01/on-kidneys_03.html

Central to normal kidney function is the filtration of small substances and retention of cells and larger molecules by the glomerular filtration barrier (GFB). The selectivity of the barrier is dependent on both the size and charge of the solutes, being particularly restrictive to negatively charged proteins. Moving from blood plasma to the Bowman's capsule, the GFB consists of three layers: glomerular endothelial cells (GEnCs), the glomerular basement membrane (GBM) and podocytes (Figure 1.2).

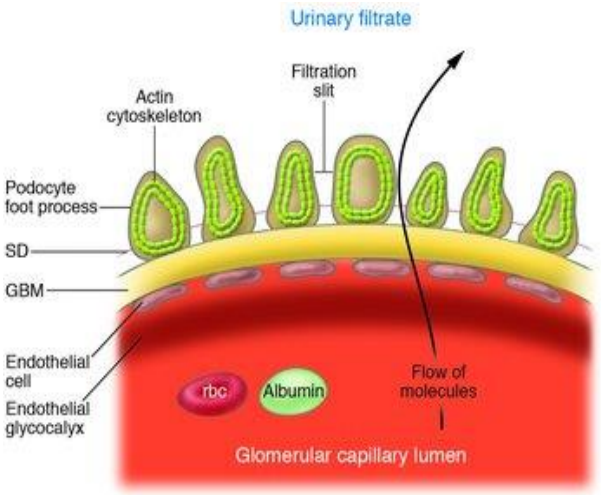


Figure 1.2: Schematic diagram of the glomerular filtration barrier

GBM, glomerular basement membrane; rbc, red blood cell; SD, slit diaphragm. Adapted with permission of American Society for Clinical Investigation from Ronco, P. (2007) [4]; permission conveyed through Copyright Clearance Center, Inc.

1.1.1 The glomerular filtration barrier

1.1.1.1 Glomerular endothelial cells

Blood enters the glomerulus via the afferent arteriole which branches into a network of multiple capillaries. These are lined by GEnCs which form a single cell layer punctuated by openings, 60-100 nm in diameter, called fenestrae, allowing rapid passage of water and solutes. Although the fenestrae are larger than plasma proteins such as albumin, it has generally been thought that movement is prevented by repulsion from the negatively-charged glycocalyx which covers the GEnCs. The glycocalyx is composed of glycoproteins, glycosaminoglycans and proteoglycans [5]. Adsorption of plasma components into the glycocalyx forms a thicker covering called the endothelial surface layer (ESL) which further narrows the fenestrae and restricts movement [6]. The importance of the ESL at preventing loss of protein into the urine (proteinuria) has

been demonstrated in animal models and humans by scenarios where the glycocalyx is lost. Adriamycin-induced glycocalyx shedding in rat models led to significant proteinuria and diabetes-associated proteinuria is correlated with ESL damage in humans [7, 8].

Normal development and function of GEnCs is dependent on cross-talk between these cells and podocytes. Studies have highlighted the importance of vascular endothelial growth factor A (VEGFA) secreted by podocytes acting in a paracrine fashion on VEGF receptors on GEnCs [9]. Blockage of signalling using anti-VEGF antibodies resulted in GEnC abnormalities and proteinuria. Both GEnCs and podocytes function together to produce the GBM.

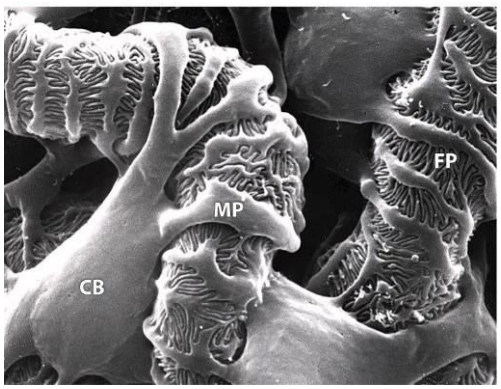
1.1.1.2 Glomerular basement membrane

The GBM forms both a scaffold to support GEnCs and podocytes and a functional component of the filtration barrier. It consists mainly of a lattice of proteins, principally collagens, laminins, heparin sulfate proteoglycans and nidogen-1 [10]. Evidence for the importance of the GBM to the GFB selective filtration function derives from genetic disease in humans. The predominant form of collagen in the mature GBM is type IV collagen, composed of trimers of α3, α4 and α5 subunits. Pathogenic variants in the respective genes (*COL4A3*, *COL4A4* and *COL4A5*) cause Alport syndrome (AS) [11]. This is characterised by abnormal thinning of the GBM, passage of blood into the urine (haematuria) and sometimes proteinuria, in addition to extra-renal features such as deafness. Laminins are also trimeric proteins which cross-link to the type IV collagen network. The predominant form is laminin-521 (composed of α5, β2 and γ1 subunits). Pathogenic variants in *LAMB2*, which encode laminin-β2, cause Pierson syndrome, an autosomal

recessive condition with congenital onset of proteinuria [12]. Laminins interact directly with podocytes, in particular with their actin cytoskeleton via other proteins such as integrins and dystroglycan, as discussed below.

1.1.1.3 Podocytes

Podocytes are highly specialised epithelial cells located on the urinary side of the GBM (Figure 1.3). Extending from their cell bodies are branching primary processes and more distal foot processes (FPs) which interdigitate with those of adjacent cells [13]. Intercellular junctions, called slit diaphragms (SDs), form structural and functional links across the 30-50 nm-wide filtration slits between the FPs of different cells. The SD is the final selective barrier to the passage of substances from the plasma before entering the urinary filtrate and its ultrastructure and molecular components have been extensively studied in recent years [3]. It has been suggested that the role of the SD is to keep FPs apart, like a "molecular spring", rather than pull them together [14]. It is now recognised to be not purely a physical barrier but, perhaps more importantly, a focus of proteins essential for signalling between FPs and internally to the podocyte cytoskeleton and nucleus.



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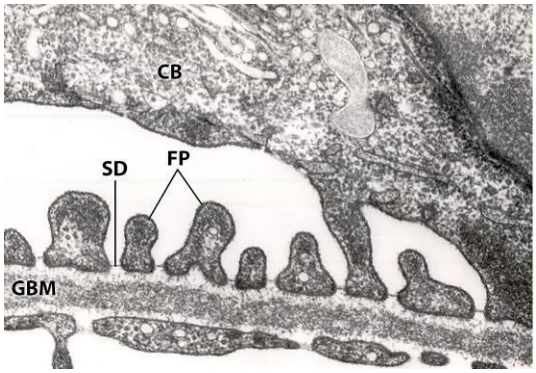


B

Figure 1.3: Ultrastructure of the glomerular filtration barrier

A: Scanning electron microscopy view of podocytes and their foot processes; B: Transmission electron microscopy view of the glomerular filtration barrier. CB, cell body; FP, foot process; GBM, glomerular basement membrane; MP, major process; SD, slit diaphragm. Republished with permission of Annual Reviews, Inc, from Greka, A. & Mundel, P. (2012) [15]; permission conveyed through Copyright Clearance Center, Inc.

Maintenance of the FP structure is dependent on its actin cytoskeleton. Normally, actin is highly organised into parallel contractile bundles centrally within the FP and a network of cortical actin lies more peripherally adjacent to the plasma membrane [16, 17]. This allows the FPs to move and regulate the filtration slits between adjacent cells. Any process, either intrinsic or extrinsic to the podocyte, which disrupts this can lead to flattening and fusion of the FPs

known as effacement. This structural change alters the integrity of the slit diaphragm and causes proteinuria, a characteristic feature of NS as discussed later [15]. The SD separates the FP surface membrane into an apical domain which faces the urinary filtrate and a basolateral domain which contacts the GBM. Signalling via proteins in either domain and at the SD can modulate the actin cytoskeleton.

One of the key proteins at the SD is nephrin, encoded by *NPHS1*. It has a large extracellular domain which contacts with neighbouring nephrin molecules. The short intracellular domain can be phosphorylated and interacts with several adaptor proteins, including Nck, to regulate the actin cytoskeleton [18]. Many other proteins which interact with or regulate the cytoskeleton are critical for normal podocyte function, and are discussed in recent reviews [3, 19]. There is an increasing list of genes, some coding for these proteins, which when mutated cause nephrotic syndrome and these will be discussed subsequently in relation to the genetics of NS.

1.1.2 Kidney function and the glomerular filtration rate

As part of its normal function, an adult kidney filters approximately 180 litres of plasma each day. This can be quantified by the glomerular filtration rate (GFR). In a human subject this can be measured formally by intravenous (IV) injection of a known quantity of inulin and measurement of its excretion into urine over a fixed period of time. Inulin is an inert protein which is freely filtered in the kidneys and is neither synthesised nor metabolised in the body. The GFR can then be calculated as volume per unit time (ml/minute). Since the number of glomeruli, and therefore GFR, is dependent on body size, and more specifically

body surface area, the calculation is usually standardised to allow comparison between individuals by reporting GFR in ml/min/1.73m^2. In clinical care, injection of inulin is an invasive and impractical method of measurement of GFR on a routine basis. The estimated GFR (eGFR) is, therefore, frequently used, which is calculated on the basis of creatinine clearance. Creatinine is a by-product of normal muscle metabolism and is generally released into plasma at a constant rate dependent on muscle bulk which is related to age, size and sex of the subject. Creatinine concentration (in mg/dL or µmol/L) can be measured on routine blood tests and there are several formulae for the calculation of eGFR based on creatinine concentration. A commonly-used method, and one used in this study, is the Schwartz formula [20]:

$$eGFR = \frac{Ht \times k}{Cr_{serum}}$$

Where Ht is height in centimetres, k is a constant (0.45 for infants 1 – 52 weeks old; 0.55 for children 1 – 13 years old; 0.55 for adolescent females 13-18 years old; and 0.7 for adolescent males 13-18 years old), and Cr_{serum} is serum creatinine in mg/dL. The constant, k, was determined empirically by Schwartz and colleagues and varies with age and gender in order to account for the differing relationship between height and muscle mass in these groups. A normal GFR is > 130 ml/min/1.73m^2. The persistent loss of normal renal filtration function is, by definition, chronic kidney disease (CKD).

Classification of CKD by stages is shown in Table 1.1.

Table 1.1 Classification of CKD by stage

CKD Stage	Glomerular filtration rate (ml/min/1.73m^2)	Comments
1	≥ 90	Kidney damage with normal or increased GFR
2	60-89	Kidney damage with mild reduction of GFR
3	30-59	Moderate reduction of GFR
4	15-29	Severe reduction of GFR
5	< 15 (or dialysis)	Kidney failure
5Tx		Transplanted

Adapted from Hogg *et al*. 2003 [21]
Legend: CKD, chronic kidney disease; GFR, glomerular filtration rate

A patient who remains persistently at CKD5 is in end-stage renal failure (ESRF) and, if untreated, the accumulation of urea and other waste substances, electrolyte disturbance and acid-base derangement would be incompatible with life. At this point, renal replacement therapy (RRT), usually in the form of dialysis, or kidney transplantation is required.

1.2 Nephrotic syndrome

1.2.1 Diagnosis and Classification

Nephrotic syndrome is diagnosed clinically by the triad of proteinuria (urine protein:creatinine ratio > 200mg/mmol or ≥ 3+ dipstick proteinuria), hypoalbuminaemia (plasma albumin < 25g/L) and oedema [22]. It is clear that NS is not a single disease but a common endpoint for various pathological processes. NS may be secondary to other conditions such as hypertension, obesity, systemic lupus erythematosus (SLE) or infections such as human

immunodeficiency virus (HIV) [23]. More frequently, however, it is primary or idiopathic in which such diseases are not identified. Currently the standard initial treatment for idiopathic nephrotic syndrome (INS) in childhood is oral steroids, usually prednisolone. Responsiveness to steroids is an important method of classification of INS used in clinical practice (Table 1.2) [22]. The definition of steroid-resistance varies between studies and publications, being failure to respond to either 4 weeks or 8 weeks of oral high-dose prednisolone [23-25]. Rarely, INS presents antenatally, at birth or within the first three months of life: this is called congenital nephrotic syndrome (CNS).

Table 1.2 Definitions and Classification in Nephrotic Syndrome

Type	**Description**
Remission	uPCR < 200 mg/g (< 20 mg/mmol) or < 1+ dipstick protein for 3 consecutive days
Relapse	uPCR ≥ 2000 mg/g (≥ 200 mg/mmol) or ≥ 3+ dipstick protein for 3 consecutive days
SSNS	Remission achieved within 4 weeks of high-dose oral prednisolone, and subsequent relapses respond to steroids
FR-SSNS	≥ 2 relapses within the first 6 months, or ≥ 4 relapses within any 12-month period
SD-SSNS	Relapse while on steroid therapy or within 2 weeks of discontinuation
SRNS	Failure to achieve remission within 4 weeks of high-dose oral prednisolone
Primary SR	Failure to achieve remission within the initial 4 weeks of high-dose oral prednisolone
Secondary SR	Remission achieved within the initial 4 weeks but subsequent relapse (sometimes months or years later) not controlled by high-dose oral steroids
Presumed SR	Presentation is with CNS or NS associated with a syndrome or in established renal failure, therefore steroids presumed to be ineffective

Adapted from [22].
Legend: CNS, congenital nephrotic syndrome; FR, frequently-relapsing; SD, steroid dependent; SR, steroid resistance; SRNS, steroid resistant nephrotic syndrome; SSNS, steroid sensitive nephrotic syndrome; uPCR, urine protein:creatinine ratio

1.2.2 Epidemiology

INS affects children of all ages with an incidence of 1 - 7 per 100,000 children/year [26]. A regional UK epidemiological study from 1987-1998 calculated an age-sex standardised incidence of 2.3 per 100,000 children/year and male:female ratio of 1.6:1 [25]. Onset is most common in the 1-4 year age group as shown in Figure 1.4.

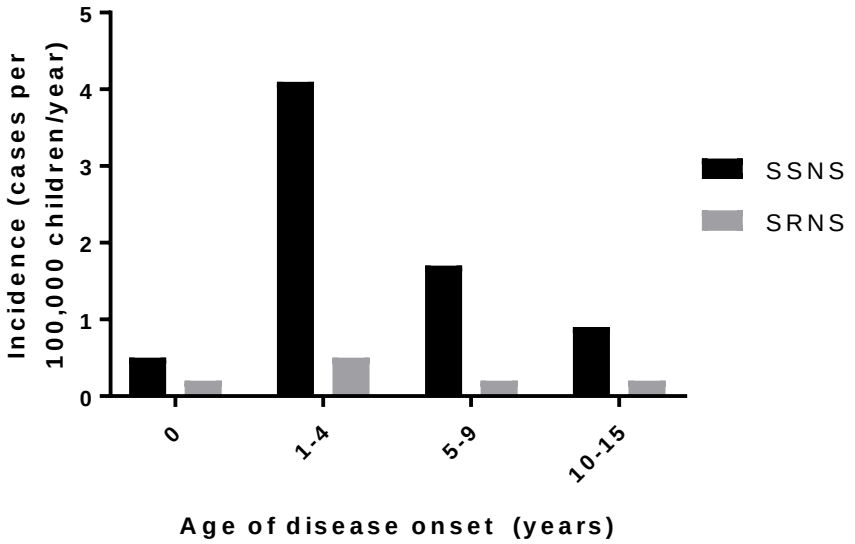


Figure 1.4: Incidence of nephrotic syndrome in Yorkshire, UK, stratified by age group and subtype

Data from McKinney *et al*. (2001) [25]

SRNS represents 10-20% of cases with a UK incidence reported of 0.3 per 100,000 children/year [25]. International studies have revealed variation of INS incidence with figures of 3.35/100,000 children/year in Paris, France, and 6.49/100,000 children/year in Japan [27, 28]. Consistent with these findings, other reports examining ethnic differences identified higher incidence of INS in South and Southeast Asians compared with Europeans [29]. Furthermore, Afro-Caribbean and Hispanic patients are more likely to have steroid-resistance, and

worse long-term outcomes, compared with White Caucasians [30]. There are suggestions that incidence of SRNS, and the most commonly-associated biopsy finding of focal segmental glomerulosclerosis (FSGS, see below), has increased over the past three decades [31-33]. Although changes in biopsy patterns may partly account for this, it is likely that the cohort of children with SRNS has grown in recent years.

1.2.3 Pathology

As part of the investigation of NS once steroid resistance is evident, the International Kidney Disease Improving Global Outcomes (KDIGO) Clinical Practice Guidelines for SRNS in Children recommend performing a renal biopsy and processing for light microscopy, immunofluorescence and electron microscopy [34]. NS is associated with various histopathological appearances on biopsy.

1.2.3.1 Minimal change disease

In SSNS, although biopsy is not routinely performed, the most common finding is minimal change disease (MCD) [35]. This is characterised by normal appearances on light microscopy with no immune complex deposits but electron microscopy (EM) reveals disruption of normal podocyte architecture with effacement and fusion of foot processes [36]. MCD is sometimes used synonymously with SSNS. While it is assumed that all (unbiopsied) patients with SSNS have MCD, not all patients with MCD are steroid-sensitive. In fact, between 3% and 35% of SRNS patients in different international studies have MCD on biopsy [37]. Historical studies, from the 1960s - 1970s in which all

patients with nephrotic syndrome underwent biopsy, showed that 95% of patients with MCD have a good outcome with no or infrequent relapses but up to 5% progress to ESRF or die earlier from complications [38].

1.2.3.2 Focal segmental glomerulosclerosis

FSGS is the single commonest finding on renal biopsy in paediatric patients with SRNS identified in 38.7% to 70.6% of cases [37, 39-42]. This is characterised by changes visible on light microscopy of scarring of glomeruli which is focal (some but not all glomeruli) and segmental (only part of each affected glomerulus). On EM, extensive foot process effacement is found but with no other abnormalities of the GBM. Both MCD and FSGS are considered primary disorders of podocytes, or podocytopathies. It is hypothesised that they lie on a pathologic spectrum with MCD sometimes progressing to FSGS. Given that, by definition, FSGS is focal, a biopsy which includes only a few glomeruli may miss the affected areas: it has been suggested that biopsies with fewer than 15 glomeruli cannot confidently exclude FSGS [43].

Other histological patterns seen in SRNS are summarised in Table 1.3 and include diffuse mesangial sclerosis (DMS), mesangioproliferative glomerulonephritis (MesPGN) and "Finnish type" CNS characteristically seen in patients with pathological variants in *NPHS1* (nephrin). It is evident that there is not direct one-to-one correspondence between the clinical presentation (steroid sensitive versus steroid resistant) and underlying renal histology. A small proportion of patients with FSGS are steroid-sensitive, although exact numbers are not clear since most SSNS patients are not biopsied. Therefore, it is unclear to what extent histology predicts response to treatment or outcome.

Table 1.3 Renal biopsy findings in children with SRNS

Biopsy Finding	Frequency (%) in SRNS*	Description
FSGS	56.0	Focal and segmental scarring, extensive foot process effacement on EM, absence of immune complex deposits
MCD	21.1	Normal glomeruli on LM; foot process effacement on EM
MesPGN	12.4	Mesangial cell proliferation and increased mesangial matrix on LM; mesangial IgM deposits on IF; granular deposits in mesangial matrix on EM
DMS	2.9	Small condensed glomeruli, increased dense mesangial collagen on LM, widespread foot process effacement on EM
MPGN	2.6	Endocapillary proliferation, double contours of GBM on silver stain on LM; IgG, C3 and C1q deposits along capillary wall or mesangium on IF; subendothelial and mesangial deposits, foot process effacement on EM
GGS	1.3	Scarring of the whole of each affected glomerulus on LM; extensive foot process effacement on EM
MN	0.8	Spikes/holes appearance of GBM on LM; IgG and C3 capillary wall deposits on IF; subepithelial deposits and foot process effacement on EM
CNS	0.6	Glomeruli may be normal or have variable mesangial and/or endocapillary hypercellularity on LM; complete foot process effacement on EM; absence of immune complex deposits
Other	2.3	

* Frequency data from the European PodoNet Registry Cohort of childhood-onset SRNS, CNS and persistent sub-nephrotic proteinuria (n = 1368) [44]. Descriptions based on [36, 45-49].
Legend: CNS, Finnish-type congenital nephrotic syndrome; DMS, diffuse mesangial sclerosis; EM, electron microscopy; FSGS, focal segmental glomerulosclerosis; GBM, glomerular basement membrane; GGS, global glomerulosclerosis; IF, immunofluorescence; LM, light microscopy; MCD, minimal change disease; MesPGN, Mesangioproliferative glomerulonephritis; MN, membranous nephropathy; MPGN, membranoproliferative glomerulonephritis

Performing a renal biopsy in children with SRNS is associated with inherent risks and costs [50, 51]:

- Technically difficult in younger children (< 2 years)
- Haemorrhage

- Arteriovenous fistula
- Pain
- Inadequate or unrepresentative sample (no renal tissue or very few glomeruli)

The key, linked questions for clinicians when considering a renal biopsy are:

1. To what extent does knowing the histology help to predict response to treatment and outcome?
2. Will the biopsy result change management?
3. Do the benefits outweigh the risks?

1.2.4 Long term outcomes and complications

Nephrotic syndrome is associated with a variety of complications: in the short term these are associated with proteinuria and loss of functional proteins from the circulation; in the long term they mostly relate to development of CKD [23]. Over a period of days, hypoalbuminaemia and reduced intravascular oncotic pressure result in fluid redistribution out of the circulation and into surrounding tissues (causing oedema) and "third spaces" such as the lungs (resulting in pleural effusions) and abdomen (leading to ascites). Loss of specific plasma proteins also have more targeted effects:

- Reduced levels of immunoglobulins predispose to infections
- Falling low density lipoprotein (LDL) and changes in enzymes in the lipid biosynthesis pathway lead to hypercholesterolaemia and elevated triglycerides
- Loss of thyroid binding globulin causes functional hypothyroidism

Long-term complications result both from the progression of the underlying disease and side effects of treatments used to control it. Patients with SSNS who remain steroid-sensitive with infrequent relapses usually have minimal long-term sequelae and do not develop ESRF. Patients with FR-SSNS or SD-SSNS are at risk of side effects from chronic steroid exposure and non-steroid immunosuppression, which will be discussed below. The greatest burden of long-term complications, however, falls on patients with SRNS. In a retrospective longitudinal study, 25% of children with SRNS progressed to ESRF within 5 years and 42% within 10 years [52].

For clinicians caring for patients with SRNS, some of the key questions are:

1. Will this patient respond to non-steroid immunosuppression and, if so, which one?
2. Will this patient progress to ESRF?
3. If this patient reaches ESRF and requires a kidney transplant, should we consider live-related donation from a family member?
4. Will this patient suffer post-transplantation disease recurrence and how best can this be treated (and, if possible, prevented)?

Research endeavours into the genetic and immunological pathogenesis of SRNS over the past two decades are making some inroads towards answering these questions. It is increasingly clear that patients with SRNS are a heterogeneous group. Fundamental to improvement in outcomes is the subcategorisation, or stratification, of patients into more homogeneous subgroups. A scientific understanding about pathogenesis can then be translated from bench to bedside by the development of targeted therapies. The following sections will

provide a review of our current understanding of pathogenesis, treatment and stratification in SRNS which forms the basis of this study.

1.3 Genetics of Nephrotic Syndrome

1.3.1 Historical background

Since the early 20th Century, babies with *in utero* oedema had been reported and rarely survived beyond a few months of life. In Finland in the 1950s, cohorts of babies were identified with characteristic changes on renal biopsy / post-mortem study which were described as congenital nephrotic syndrome of the Finnish type [53]. The antenatal onset of severe disease, together with the familial inheritance pattern in siblings (boys and girls) born to unaffected parents, strongly suggested an autosomal recessive pattern of genetic transmission. The use of family studies with linkage disequilibrium analysis narrowed the search to genes on chromosome 19 [54]. Subsequent positional cloning identified mutations in the gene later named *NPHS1* (for "nephrotic syndrome 1") as being the cause of disease in these children [55]. Two years later in 2000 the second gene, *NPHS2*, was also recognised as an autosomal recessive cause of SRNS [56]. The number of causative genes has rapidly expanded in the past 5 years and now numbers over 50, most of which are structurally or functionally related to the podocyte. These are summarised in Table 1.4 below.

SRNS is a genetically heterogeneous disorder with dominant, recessive or sporadic inheritance with a single-gene cause reported in 29.5% of cases [57]. There is a differing spectrum of genes and mutations associated with congenital and childhood-onset disease in comparison with adult disease. Genetic mutations

may manifest as a renal-only phenotype or nephrotic disease as part of a wider syndrome [58]. The proportion of cases with an identified genetic cause is inversely related to the age of disease onset: 69.4% - 100% of patients with CNS have been reported as having a genetic condition [57, 59]. Genes with variants inherited in a recessive pattern include *NPHS1* (nephrin) [55], *NPHS2* (podocin) [56], *LAMB2* (laminin subunit β2) [12] and *PLCE1* (phospholipase C.ε1) [60]. Those following a dominant pattern include *INF2* (inverted formin-2)[61], *TRPC6* (transient receptor potential channel 6) [62], *ACTN4* (actinin-α4) [63], *LMX1B* (LIM homeobox transcription factor 1-β) [64] and *WT1* (Wilms tumour) [65]. Pathogenic variants in certain genes are associated with multisystem syndromes for example Pierson syndrome (*LAMB2*), Denys-Drash syndrome (*WT1*) and Charcot-Marie Tooth disease (*INF2*).

The phenotypic spectrum is widening, both in terms of age of onset [66] and phenotypic variability, with novel phenotypes for individual genes emerging [67]. Recent evidence indicates that the phenotype of a mutation in an SRNS gene can be modified by a mutation in one of the collagen genes (*COL4A3, COL4A4, COL4A5*) [68] and potentially other SRNS genes. The phenotype can also depend upon the location of the mutation within the gene/protein [69], and whether one or two mutations are present in a single gene.

1.3.2 *NPHS1*

NPHS1 codes for nephrin, which, as discussed previously, is a key protein at the podocyte slit diaphragm. Mutations in this gene are the commonest cause for CNS, being responsible for 40-60% of cases. In those of Finnish heritage, over 90% of CNS results from two founder mutations which result in a premature stop

codon and absence of nephrin at the SD [70, 71]. Inheritance follows an autosomal recessive pattern and children usually present within the first 3 months of life, progressing to ESRF within the first few years. Since first being identified in Finnish infants, causative mutations in *NPHS1* have been detected in patients with onset in older childhood (5 months – 8 years) and in adulthood [57, 66, 72]. Over 240 mutations in *NPHS1* have been identified in international populations [73, 74] and the phenotypic spectrum is widening to include patients with homozygous mutations who respond to immunosuppressive therapy and do not progress to ESRF.

1.3.3 *NPHS2*

NPHS2 is the gene which is most-frequently implicated in genetic forms of SRNS and encodes the protein podocin [56]. One study identified it as the cause in 39.1% of CNS and 35.3% of infantile onset NS [75]. Podocin is localised to the SD, in particular to lipid rafts which are functionally-important signalling microdomains in the plasma membrane [76]. It recruits and stabilises nephrin as well as increasing its activity. Mutations in *NPHS2* have been shown to disrupt the targeting of nephrin to the lipid raft microdomains [77]. Homozygous or compound heterozygous pathogenic variants cause onset of SRNS usually under the age of 6 years with FSGS found on renal biopsy. Within European populations, the R138Q variant is the most common although over 125 different mutations have been identified. The R229Q variant is recognised as a non-neutral polymorphism: in a homozygous state it does not cause disease however it is pathogenic when in compound heterozygosity with specific variants in exons 8 or

9 on the other allele [69]. Typically, children with podocin mutations progress rapidly to ESRF.

1.3.4 WT1

WT1 encodes Wilms tumour 1, a nuclear transcription factor which controls development of the kidney and urogenital tract and affects expression of several genes including *NPHS1* [78, 79]. Unlike *NPHS1* and *NPHS2*, *WT1* mutations are inherited in an autosomal dominant fashion (mostly in exons 8 or 9 of *WT1*) and are often associated with syndromic presentation with extra-renal features. Denys-Drash syndrome (DDS) has features of infantile-onset SRNS with DMS on renal biopsy, ambiguous genitalia and Wilms tumour [80]. Frasier syndrome (FS) is notable for the association of SRNS with male pseudohermaphroditism. These features highlight the importance of confirmation of karyotype (XX or XY) in patients with suspected or confirmed *WT1* mutations. In addition to the pattern of extra-renal features, the pattern of kidney disease (age of onset, progression to ESRF) appears to be associated with the specific location of the mutation within the gene (exons, introns, splice sites) [80, 81]. Although many patients with *WT1* mutations progress to ESRF, there have been reports of a small number of patients responding to intensified immunosuppressive (IIS) therapy with ciclosporin and IV steroids possibly via direct effects on podocytes rather than immune system modulation [82]. The non-immunologic mechanisms of action of immunosuppressive drugs are discussed further in Section 1.5.1.

1.3.5 Collagen genes

As discussed earlier, collagen IV is a major structural component of the GBM and mutations in *COL4A3*, *COL4A4* and *COL4A5* cause classical Alport syndrome, with clinical features of haematuria and sensorineural hearing loss together with typical EM findings on renal biopsy of diffuse GBM lamellation. However, several patients have been reported presenting with FSGS and no extra-renal features who are found to have probably pathogenic variants in collagen genes on subsequent testing [83, 84].

1.3.6 Other genes

Over 50 genes have been identified which are thought to cause inherited forms of SRNS and are summarised in Table 1.4 and discussed further in several recent reviews [58, 85]. Within the group of patients with genetic SRNS there is great heterogeneity in terms of age of disease onset, potential response to medication and progression to ESRF. Knowledge of which is the causative gene, and the specific mutation within that gene, will assist with stratification of patients as discussed further in Chapters 2 and 4.

Large national and international studies have been completed in recent years which have undertaken parallel testing of between 8 and 28 SRNS/FSGS-associated genes in almost 4000 patients (Table 1.5). Each has identified a genetic cause in around 30% of the patients tested. Although there are likely to be more pathogenic genes to be identified and some patients' disease may be caused by combinatorial effects of two or more genes [68, 86], our current understanding is that the majority of patients with SRNS have a non-genetic aetiology.

Chapter 2 will explore genotype-phenotype associations in a UK cohort of 187 patients all of whom had genetic analysis by whole exome sequencing. The real-world utility of clinical testing using a next-generation-sequencing gene panel will be examined in Chapter 4.

Table 1.4: Genes associated with SRNS

Gene	Protein	Inheritance	Disease association	Key reference (First author, year)
Slit diaphragm				
NPHS1	Nephrin	AR	CNS / SRNS	Kestilä 1998 [55]
NPHS2	Podocin	AR	CNS / SRNS	Boute 2000 [56]
CD2AP	CD2-associated protein	AD/AR	FSGS / SRNS	Löwik 2007 [87]
PLCE	Phospholipase Cε1	AR	CNS / SRNS	Hinkes 2006 [60]
PTPRO	Protein tyrosine phosphatase, receptor type O	AR	NS	Ozaltin 2011 [88]
TRPC6	Transient receptor potential channel 6	AD	Familial and sporadic SRNS (mainly adults)	Winn 2005 [62]
MAGI-2	Membrane associated guanylate kinase, WW and PDZ domain containing 2	AR	CNS/SRNS	Bierzynska 2017 [89]
CRB2	Crumbs 2, cell polarity complex component	AR	FSGS/SRNS	Ebarasi 2015 [90]
Nuclear proteins				
WT1	Wilms' tumour 1	AD	Sporadic SRNS (children—may be associated with abnormal genitalia); Denys-Drash and Frasier syndrome	Pelletier 1991 [65]
SMARCAL1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A-Like 1	AR	Schimke immuno-osseous dysplasia	Boerkoel 2002 [91]
LMX1B	Lim homeobox transcription factor 1β	AD	Nail patella syndrome; also, FSGS without extrarenal involvement	Dreyer 1998 [64]
E2F3	E2F transcription factor 3	AD	FSGS + mental retardation (whole gene deletion)	Izu 2011 [92]

Gene	Protein	Inheritance	Disease association	Key reference (First author, year)
NXF5	Nuclear RNA export factor 5	X-linked recessive	NS, cosegregating with heart block	Esposito 2013 [93]
PAX2	Paired Box 2	AR	FSGS, CAKUT	Barua 2014 [94]
WDR73	WD repeat domain 73	AR	Galloway-Mowat syndrome, microcephaly, SRNS	Colin 2014 [95]
NUP93	Nucleoporin 93	AR	FSGS/SRNS, haematuria	Braun 2016 [96]
NUP107	Nucleoporin 107	AR	FSGS/SRNS	Miyake 2015 [97]
NUP205	Nucleoporin 205	AR	FSGS/SRNS	Braun 2016 [96]
XPO5	Exportin 5	AR	FSGS/SRNS	Braun 2016 [96]
Actin cytoskeleton and Signalling				
ACTN4	α-Actinin-4	AD	Familial and sporadic SRNS (usually adult)	Kaplan 2000 [63]
INF2	Inverted formin-2	AD	Familial and sporadic SRNS, FSGS-associated Charcot-Marie-Tooth disease	Brown 2010 [61]
MYH9	Myosin heavy chain 9	AD, association	MYH9-related disease; Epstein and Fechtner syndromes	Kopp 2008 [98]
SYNPO	Synaptopodin	Uncertain	Adult-onset NS	Asanuma 2005, Huber 2006 [99, 100]
APOL1	Apolipoprotein L1	Risk factor	Increased susceptibility to FSGS in African Americans and those of African ancestry	Genovese 2010 [101]
MYO1E	Myosin 1E	AR	Familial SRNS	Mele 2011, Sanna-Cherchi 2011 [102, 103]
ARHGAP24	Rho GTPase-activating protein 24	AD	FSGS	Akilesh 2011 [104]
ARHGDIA	Rho GDP-dissociation inhibitor-1	AR	CNS	Gupta 2013 [105]
MED28	Mediator complex subunit 28	AR	NS	Fang 2012 [106]
ANLN	Anillin actin binding protein	AD	Adult-onset FSGS	Gbadegesin 2014 [107]

Gene	Protein	Inheritance	Disease association	Key reference (First author, year)
EMP2	Epithelial membrane protein 2	AR	SSNS, SRNS	Gee 2014 [108]
CUBN	Cubilin	AR	NS, Megaloblastic anaemia	Ovunc 2011 [109]
PODXL	Podocalyxin like	AD	NS	Takeda 2001 [110]
TTC21B	Tetratricopeptide Repeat Domain 21B	AR	NS, FSGS, nephronophthisis, tubulointerstitial lesions	Huynh Cong 2014 [111]
KANK1, *KANK2*, *KANK4*	Kidney Ankyrin repeat-containing protein	AR	SSNS, SRNS	Gee 2015 [112]
SGPL1	Sphingosine-1-phosphate lyase 1	AR	CNS, hypogonadism, adrenal calcification	Janecke 2017, Lovric 2017 [113, 114]
FAT1	FAT atypical cadherin 1	AR	NS, haematuria, tubular ectasia, intellectual impairment	Gee 2016 [115]
CTLA4	Cytotoxic T-lymphocyte associated protein 4	Risk factor	Sporadic NS, MCD	Spink 2013 [116]
Mitochondrial				
MTTL1	Mitochondrial tRNA leucine 1	Mitochondrial	SRNS / FSGS, MELAS, hearing loss, diabetes mellitus	Löwik 2005 [117]
COQ2	Coenzyme Q_2	AR	Mitochondrial disease, encephalopathy / isolated nephropathy	Diomedi-Camassei 2007 [118]
COQ6	Coenzyme Q_6	AR	NS +/- sensorineural deafness; DMS	Heeringa 2011 [119]
COQ7	Coenzyme Q_7, hydroxylase	AR	Mitochondrial disease, encephalopathy	Freyer 2015 [120]
COQ9	Coenzyme Q_9	AR	Mitochondrial disease, encephalopathy, renal tubulopathy	Duncan 2009 [121]
ZMPSTE24	Zinc metallopeptidase STE24	AR	Mandibuloacral dysplasia with FSGS	Agarwal 2006 [122]
PDSS2	Decaprenyl diphosphate synthase subunit 2	AR	Leigh syndrome	López 2006 [123]

Gene	Protein	Inheritance	Disease association	Key reference (First author, year)
ADCK4	aarF domain containing kinase 4	AR	SRNS / FSGS	Ashraf 2013 [124]
CYP11B2	Cytochrome P450 family 11 subfamily B member 2	Association	Corticosterone methyloxidase deficiency, Familial hyperaldosteronism	Bantis 2011 [125]
Glomerular basement membrane				
LAMB2	Laminin subunit beta 2	AR	Pierson syndrome	Zenker 2004 [12]
ITGA3	Integrin subunit alpha 3	AR	Interstitial lung disease, CNS, and mild epidermolysis bullosa	Has 2012 [126]
ITGB4	Integrin subunit beta 4	AR	Epidermolysis bullosa and pyloric atresia, FSGS	Kambham 2000 [127]
GPC5	Glypican 5	Risk factor	Increased risk of NS	Okamoto 2011 [128]
COL4A3	Collagen type IV alpha 3 chain	AR	Alport syndrome	Malone 2014, Gast 2016 [83, 129]
COL4A4	Collagen type IV alpha 4 chain	AR	Alport syndrome	Malone 2014, Gast 2016 [83, 129]
COL4A5	Collagen type IV alpha 5 chain	X-linked	Alport syndrome	Gast 2016 [129]
CD151	CD151	AR	NS, pretibial bullous skin lesions, neurosensory deafness, bilateral lacrimal duct stenosis, nail dystrophy, and thalassemia minor	Karamatic Crew 2004 [130]
Metabolic				
ALG1	ALG1, chitobiosyldiphosphodolichol beta-mannosyltransferase	AR	Congenital disorder of glycosylation	Park 2016 [131]
PMM2	Phosphomannomutase 2	AR	Congenital disorder of glycosylation	Van der Knapp 1996 [132]
Lysosomal				
SCARB2 /	Lysosome membrane protein	AR	Action myoclonus-renal failure syndrome +/- hearing loss	Berkovic 2008 [133]

Gene	Protein	Inheritance	Disease association	Key reference (First author, year)
LIMP2	2			
ALMS1	ALMS1, centrosome and basal body associated protein	AR	Alström syndrome, retinitis pigmentosa, sensorineural hearing loss	Marshall 2015 [134]
Other				
DGKE	Diacylglycerol kinase ε	AR	Atypical HUS, SRNS	Bierzynska 2017, Lemaire 2013 [1, 135]
OCRL	OCRL, inositol polyphosphate-5-phosphatase	X-linked recessive	Lowe syndrome, Dent disease type 2	De Mutiis 2015 [136]

Legend: AD, autosomal dominant; AR, autosomal recessive; CAKUT, congenital abnormalities of the kidney and urinary tract; CNS, congenital nephrotic syndrome; DMS, diffuse mesangial sclerosis; FSGS, focal segmental glomerulosclerosis; HUS, haemolytic uremic syndrome; Kb, kilobases; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; NS, nephrotic syndrome; SDNS, steroid-dependent nephrotic syndrome; SRNS, steroid-resistant nephrotic syndrome; SSNS, steroid-sensitive nephrotic syndrome

Table 1.5: National and international studies genotyping patients with SRNS

		Santín 2011 [59]	**McCarthy 2013 [137]**	**Bullich 2014 [68]**	**Sadowski 2014 [57]**	**Trautmann 2015 [44]**	**Büscher 2016† [138]**	**Ogino 2016 [139]**	**Wang 2017 [140]**
Phenotype		CNS, primary FSGS/DMS, SRNS (primary SR)	SRNS/FSGS	SRNS/FSGS	SRNS	CNS, SRNS, persistent proteinuria	CNS, SRNS	SRNS/FSGS	SRNS/FSGS, persistent proteinuria
Country		Spain	UK	Spain	International (>29 countries)	International (21 countries)	Germany	Japan	China
Genes tested		8 genes	24 genes	26 genes	27 genes	Variable, dependent on practice in each centre	10-gene panel used for 68 patients. Variable in others	26 genes	28 genes
Total patients		125	36	25 (discovery cohort only)	2016 (1783 families)	1655	231 (219 families)	24	110 (SRNS), 10 (proteinuria)
Male (%)		n/a	16 (44.4)	n/a	1067 (52.9)	n/a	106 (45.9)	12 (50.0)	67 (55.8)
Age at onset in years (%)	0-0.25	15 (12.0)	5 (13.9)	5 (20.0)	253 families (14.2)	98 (6.3)	62 (26.8)	1 (4.2)	12 (10.0)
	0.25-1	8 (6.4)	1 (2.8)	4m-5y: 9 (36.0)	n/a	106 (6.9)	n/a	0 (0)	18 (15.0)
	1-12	37 (29.6)	26 (72.2)	6-12y: 3 (12.0)	n/a	1136 (73.6)	n/a	23 (95.8)	88 (73.3)
	13-18	9 (7.2)	4 (11.1)	0 (0)	n/a	204 (13.2)	n/a	0 (0)	2 (1.7)
	> 18	56 (44.8)	0 (0)	8 (32.0)	n/a	0 (0)	n/a	0 (0)	0 (0)
	Other information	Median 14.0y; range 0-56y			Median 3.4y; range 0-63y	Unknown: 111	Median 5.1y (IQR 2.7-8.6) in NG. Median 3.5y (IQR 1.3-		Median 4.0y; range 0-17.3y

		Santín 2011 [59]	McCarthy 2013 [137]	Bullich 2014 [68]	Sadowski 2014 [57]	Trautmann 2015 [44]	Büscher 2016† [138]	Ogino 2016 [139]	Wang 2017 [140]
							8.0) in G.		
Family history positive / number with data available (%)		39/125 (31.2)	10/36 (27.8)	11/25 (44.0)	n/a	25.6%	n/a	6/24 (25.0)	19/108 (17.6)
Consanguinity / number with data available (%)		7/125 (5.6)	n/a	4 (16.0)	372 families (20.9)	28.6%	n/a	0 (0)	0 (0)
Ethnicity (% of patients where data available)	White	n/a	22 (73.3)	n/a	n/a	1151 (90.3)	n/a	0 (0)	0 (0)
	Indian	n/a	5 (16.7)	n/a	n/a	9 (0.7)	n/a	0 (0)	0 (0)
	Black African / Caribbean	n/a	0 (0)	n/a	n/a	3 (0.2)	n/a	0 (0)	0 (0)
	Pakistani	n/a	2 (6.7)	n/a	n/a		n/a	0 (0)	0 (0)
	Hispanic	n/a	0 (0)	n/a	n/a	93 (7.3)	n/a	0 (0)	0 (0)
	East Asian	n/a	0 (0)	n/a	n/a	5 (0.4)	n/a	24 (100) Japanese	0 (0)
	Native American	n/a	0 (0)	n/a	n/a	2 (0.2)	n/a	0 (0)	0 (0)
	Chinese	n/a	0 (0)	n/a	n/a		n/a	0 (0)	120 (100)
	Mixed	n/a	1 (3.3)	n/a	n/a	11 (0.9)	n/a	0 (0)	0 (0)

		Santín 2011 [59]	McCarthy 2013 [137]	Bullich 2014 [68]	Sadowski 2014 [57]	Trautmann 2015 [44]	Büscher 2016† [138]	Ogino 2016 [139]	Wang 2017 [140]
	No ethnicity data available	n/a	6	n/a	n/a	381	n/a	0	0
Biopsy findings (% of patients where data available)	FSGS	108 (93.1)	n/a	n/a	n/a	752 (56.0)	119 (58.9)	22 (100.0)	42 (53.2)
	MCD	2 (1.7)	n/a	n/a	n/a	283 (21.1)	39 (19.3)	0 (0)	16 (20.3)
	Mesangio-proliferative GN	0 (0)	n/a	n/a	n/a	166 (12.4)	0 (0)	0 (0)	7 (8.9)
	DMS	3 (2.6)	n/a	n/a	n/a	39 (2.9)	26 (12.9)	0 (0)	0 (0)
	Finnish type	3 (2.6)	n/a	n/a	n/a	8 (0.6)	8 (4.0)	0 (0)	0 (0)
	Other	0 (0)	n/a	n/a	n/a	94 (6.9)	10 (5.0)	0 (0)	14 (17.7)
	No biopsy data available / Not biopsied	9	n/a	*	*	287	29	0	41
ESRD (% of patients where data available)		69 (55.2)	12 (34.3)	n/a	n/a	25.9%	125 (54.1)	16 (66.7)	16 (15.7)
Age at ESRD in years (Mean ± SD; Median; Range)		27.3 ± 19.1; 24.0; 0.2-61.0	7.0 ± 3.9; 6.3; 2.0-13.5	n/a	n/a	n/a	n/a	12.8 ± 9.7; 10.7; 2.2-42.0	n/a

		Santín 2011 [59]	**McCarthy 2013 [137]**	**Bullich 2014 [68]**	**Sadowski 2014 [57]**	**Trautmann 2015 [44]**	**Büscher 2016[†] [138]**	**Ogino 2016 [139]**	**Wang 2017 [140]**
Kidney transplants (% of patients where data available)		53 (42.4)	8 (22.9)	n/a	n/a	14.2%	105 (45.9)	11 (45.8)	n/a
Total patients with pathogenic mutations (%)		37/110 unrelated cases (**33.6**)	11 (**30.6**) (8 families)	9 (**36.0**)	615 (**30.5**) 526 families (29.5)	277/1174 (**23.6**)	131 (**56.7**)	8 (**33.3**)	34 (**28.3**)
NPHS1		15 (13.6)	5 (13.9)	4 (16.0)	131 (7.4)	138/1088 (12.7)	35	0 (0)	7 (5.8)
NPHS2		13 (11.8)	3 (8.3)	2 (8.0)	177 (9.9)	41/208 (19.7)	43	1 (4.2)	4 (3.3)
WT1		5 (4.5)	0 (0)	1 (4.0)	85 (4.8)	48/902 (5.3)	33	2 (8.3)	7 (5.8)
PLCE1		0 (0)	1 (2.8)	0 (0)	37 (2.1)	10/75 (13.3)	2	0 (0)	1 (0.8)
LAMB2		-	0 (0)	0 (0)	20 (1.1)	5/84 (6.0)	3	1 (4.2)	1 (0.8)
TRPC6		3 (2.7)	0 (0)	1 (4.0)	9 (0.5)	1/96 (1.0)	5	1 (4.2)	1 (0.8)
INF2		1 (0.9)	0 (0)	1 (4.0)	9 (0.5)	4/112 (3.6)	3	1 (4.2)	0 (0)
SMARCAL1		-	0 (0)	0 (0)	16 (0.9)	12/68 (17.6)	-	0 (0)	1 (0.8)
COQ6		-	-	0 (0)	8 (0.4)	3/30 (10.0)	0	0 (0)	0 (0)
IGTA3		-	-	0 (0)	5 (0.3)	-	-	-	0 (0)
MYO1E		-	0 (0)	0 (0)	5 (0.3)	2/48 (4.2)	-	0 (0)	0 (0)
CUBN		-	-	0 (0)	5 (0.3)	-	-	-	2 (1.7)
COQ2		-	1 (2.8)	0 (0)	4 (0.2)	1/56 (1.8)	-	0 (0)	0 (0)
LMX1B		-	0 (0)	0 (0)	4 (0.2)	1/27 (3.7)	2	0 (0)	2 (1.7)
ADCK4		-	-	-	3 (0.2)	1/27 (3.7)	-	-	8 (6.7)
DGKE1		-	-	-	2 (0.1)	-	-	-	0 (0)

		Santín 2011 [59]	McCarthy 2013 [137]	Bullich 2014 [68]	Sadowski 2014 [57]	Trautmann 2015 [44]	Büscher 2016† [138]	Ogino 2016 [139]	Wang 2017 [140]
PDSS2		-	0 (0)	-	2 (0.1)	0/56 (0)	-	0 (0)	0 (0)
ARHGAP24		-	-	0 (0)	1 (0.06)	-	-	0 (0)	0 (0)
ARHGDIA		-	-	-	1 (0.06)	-	1	-	0 (0)
CFH		-	-	0 (0)	1 (0.06)	-	-	-	0 (0)
ITGB4		-	-	-	1 (0.06)	-	-	0 (0)	0 (0)
PTPRO		-	0 (0)	0 (0)	0 (0)	6/45 (13.3)	-	0 (0)	0 (0)
ACTN4		0 (0)	0 (0)	0 (0)	0 (0)	0/59 (0)	1	0 (0)	0 (0)
CD2AP		0 (0)	0 (0)	0 (0)	0 (0)	0/56 (0)	0	2 (8.3)	0 (0)
COL4A3		-	0 (0)	0 (0)	-	-	-	-	-
COL4A4		-	1 (2.8)	0 (0)	-	-	-	-	-
COL4A5		-	0 (0)	0 (0)	-	-	-	-	-
Others		-	-	-	-	-	3 "novel podocyte genes" (unpublished)	**	-

Legend: G, genetic patients; n/a, not available; NG, non-genetic patients; –, gene not tested in the cohort

* Biopsy data only available for patients found to have genetic disease

† Some of the cases reported in Büscher *et al.* (2016) were also included in Trautmann *et al.* (2015). Data from Büscher *et al.* (2010) [141] are not included in the above table due to the overlap of patients with the more recent study by the same authors.

** This study also analysed the following genes and found no pathogenic variants: *MYH9*, *MT-TL1*, *SCARB2*, *ZMPSTE24*, *PMM2*, *ALG1*, *GPC5*, *APOL1*.

1.4 The Circulating Factor Hypothesis

Based on recent large cohort studies (Table 1.5), approximately 70% of patients with SRNS have non-genetic disease. In this group, the prevailing hypothesis has been that the disease is driven by (an) immune-mediated circulating factor(s).

1.4.1 Evidence suggesting existence of circulating factors

The potential of circulating factors as causative agents in some cases of SRNS has been considered since the 1970s. There are several strands of evidence which support the hypothesis:

1. In 30-50% of cases, the observation of recurrence of proteinuria within minutes to hours after transplantation of a healthy donor kidney into a patient with SRNS accompanied by typical biopsy findings of podocyte effacement [142].
2. A case of rapid disease recurrence and histological changes in a kidney transplanted into a patient with SRNS but recovery of normal function and histology when removed and re-transplanted into a patient with CKD secondary to diabetes mellitus [143].
3. The case of a neonate born to a mother with NS/FSGS who had nephrotic proteinuria at birth but which resolved over subsequent days [144]. This would suggest the factor(s) can cross the placenta and is not IgG which persists in an infant's circulation for several months.
4. The effectiveness of plasmapheresis in management of post-transplant recurrence, thought to be by removal of a putative permeability factor(s) [142].

5. The reduced risk of post-transplant recurrence by repeated plasmapheresis before transplantation [145].
6. Increased permeability to albumin in isolated rat glomeruli perfused with plasma from patients with FSGS but not with control plasma [146].

1.4.2 Role of the immune system

Shalhoub originally proposed in 1974 that MCD was the result of T cell dysfunction producing a lymphocyte-derived permeability factor [147]. This was based on several observations:

- Absence of immune complexes in glomeruli
- Association of MCD with Hodgkin's disease, in which adaptive immunity is impaired
- Remission following measles infection, which modifies cell-mediated immunity
- Rapid response to corticosteroids.

The potential role of the immune system in the pathogenesis of SRNS has been supported by the fact that patients respond to various types of immunosuppressive treatment [147, 148]. Those with secondary SR, by definition, initially responded to corticosteroids and those with either primary or secondary SR can show resolution of proteinuria with drugs such as ciclosporin or tacrolimus. However, more recent research has highlighted that steroids, ciclosporin and tacrolimus can have a direct effect on podocytes and the actin cytoskeleton [149, 150].

If the immune system is involved in SRNS pathogenesis, it would seem to be by secretion of soluble factors and not by direct cellular infiltration of the glomerulus or antibody deposition at the filtration barrier. This is evidenced by the absence of neutrophils/lymphocytes and immune complex deposits on light microscopy and immunofluorescence of renal biopsies in MCD and FSGS [36, 45]. Early studies on lymphocytes from patients with MCD stimulated with concanavalin A (which acts as a T cell mitogen) showed that the supernatant contained a factor which modified vascular permeability [151]. Several T lymphocyte-derived cytokines, such as interleukin-13 (IL-13) and tumour necrosis factor alpha (TNFα) have been suggested as circulating factors in NS and are discussed below (Section 1.4.3).

Mechanisms exist by which podocytes may interact directly with the immune system in pathogenesis of NS. In order for T lymphocytes to be activated, they generally require antigen to be processed and presented by antigen-presenting cells (APCs) in the major histocompatibility complex (MHC) alongside co-stimulation. This co-stimulation is provided by direct cell-cell signalling via surface proteins. CD80 and CD86 (also known as B7.1 and B7.2) and CD40 on APCs interact with CD28 and CD40 ligand (CD40L) respectively on T cells leading to their activation [152]. Interaction of CD80 and CD86 with CTLA4 (cytotoxic T-lymphocyte-associated protein 4) on T cells, in contrast, provides an inhibitory signal. Several studies, in various disease models including MCD, have shown increased podocyte expression of CD80, increased urinary excretion of CD80 and proteinuria [153]. A report has suggested that podocytes may act as professional APC and have a more direct role in immune system function than was previously thought [154]. Further discussion of the podocyte-

immune system interaction is beyond the scope of this project but has been recently reviewed [152, 153, 155].

1.4.3 Putative circulating factors

Early studies separated FSGS plasma using fractionation columns, galactose affinity chromatography and mass spectrometry and tested for permeability-inducing activity on rat glomeruli [146]. Savin *et al*. concluded that the circulating factor has a size of < 30 kDa and identified a potential circulating factor as cardiotrophin-like cytokine factor-1 (CLCF-1). A second candidate intensively investigated by Reiser and colleagues is soluble urokinase plasminogen activator receptor (suPAR) [156]. Several studies have proposed that the factor is a protease, including activated hemopexin [157, 158]. Evidence for and against this and other putative factors is summarised in Table 1.6.

Table 1.6: Putative causative circulating factors in SRNS

Putative circulating factor	***In vitro* evidence**	***In vivo* evidence**	**Human / Clinical evidence**	**Concerns / evidence against**
suPAR	Activates podocyte $\alpha_v\beta_3$-integrin → cytoskeletal reorganisation [156] Suppresses *WT1* → reduced nephrin expression [159]	Administration causes albuminuria in uPAR-/- mice [156]	High plasma concentrations in patients with FSGS [156] High plasma concentrations in post-transplant recurrence [156]	Non-specific: raised levels in primary FSGS and other proteinuric diseases [160, 161] Inversely correlated to eGFR in various disease groups [162] Podocyte $\alpha_v\beta_3$-integrin stimulation also seen in diabetic kidney disease [163] Non-standard mouse model: administration does not cause albuminuria in WT mice [164] Different suPAR isoforms not detected by current ELISA kits [165]
CLCF-1	Increases permeability to albumin in isolated rat glomeruli, blocked by anti-CLCF-1 Ab [166] Decreases nephrin expression and disrupts podocyte cytoskeleton [166] Inhibitors of Jak/Stat3 block CLCF-1 and FSGS sera effects [167] Binds to galactose columns; galactose blocks increase in glomerular permeability by FSGS sera [146]	Administration increases glomerular permeability and proteinuria in mice [167]	Levels in FSGS patients > 100-fold higher than controls [166]	Difficult to measure – not yet tested in large patient cohorts or in other diseases
Galactose (inhibitor of circulating factor)	Blocks increase in permeability to albumin in isolated rat glomeruli caused by FSGS sera [168]			Conflicting results in clinical studies: 3/7 patients in clinical study of galactose had 50% reduction in proteinuria with stable GFR [169, 170]

CD40 autoantibodies	Disrupts podocyte cytoskeleton [171]	Co-administration with suPAR → proteinuria in WT mice but not CD40-/- mice or WT mice given CD40-blocking Ab [171]	Identified as strongest predictor of post-transplant recurrence in serum autoantibody panel [171]	Injection into WT mice did not cause proteinuria (only with suPAR) Size ~ 150kDa (larger than 30-50kDa identified as active fraction of FSGS sera) [172]
Hemopexin	Causes nephrin-dependent actin cytoskeleton reorganisation in cultured human podocytes [173]	Induces reversible proteinuria in rats [174]	Increased hemopexin activity in plasma from MCD relapse patients vs MCD remission [158]	
TNFα	Blocking TNFα pathway reverses activation of podocyte β3-integrin caused by plasma from patients with FSGS recurrence [175]	Infusion → albuminuria in rats [176]	Increase in PBMC mRNA expression in patients with MCD relapse [177] Remission of proteinuria after infliximab or adalimumab (anti-TNF mAb) treatment in some patients [178, 179]	Lack of consistent effect of anti-TNFs
Interleukin-13		Over-expression in rats → MCD-like disease and nephrotic proteinuria [180]	Increased $CD4^+$ and $CD8^+$ T lymphocyte mRNA expression in patients with MCD relapse [181]	Other conditions associated with increased IL-13 (asthma, psoriasis, allergic dermatitis) not linked to proteinuria [153]

Ab, antibody; CLCF-1, cardiotropin-like cytokine factor-1; FSGS, focal segmental glomerulosclerosis; Jak, Janus kinase; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; Stat, signal transducer and activator of transcription; suPAR, soluble urokinase type plasminogen activator receptor; TNFα, tumour necrosis factor alpha; WT, wild type
Adapted and summarised from [146, 152, 153, 182-186]

Although research to identify the putative circulating factor(s) has been undertaken for decades and is ongoing, it remains elusive [152, 182]. It may be the *presence* of a factor(s) which causes recurrent podocyte effacement and proteinuria post-transplant but also possible is the *absence* of a factor(s) which normally prevent this process. The absence of a protective factor could explain the observations discussed previously in Section 1.4.1 only if there was a permeability factor(s) which could be regulated by it. Variation in the methodology used in different studies to define the characteristics of putative circulating factors may be one reason for some lack of cross-validation of results between research groups. Furthermore, patients included in such studies may not be homogeneous and are likely receiving different medications [186]. Recently, the following criteria have been proposed to establish the causality of a putative circulating factor (quoted from Maas et al. 2014) [185]:

- The permeability factor must have biological effects *in vitro* and *in vivo*, and be confirmed in validation studies
- Identification of the permeability factor in well-phenotyped patients but not in appropriate controls and validation in independent patient cohorts
- Temporal relation of the permeability factor with disease activity and remission
- Specific removal or inhibition of the permeability factor *in vivo* blocks the biologic effect

Much of the evidence for individual circulating factors (suPAR, CLCF-1, CD40 autoantibodies) derives from the work of single research groups and, thus far, none consistently fulfils all criteria proposed by Maas *et al*. Given the heterogeneity of NS/SRNS, it may be that there is not a single permeability factor

but different factors causing disease in different non-genetic patients. Research is ongoing with the aim of providing better understanding of disease pathogenesis. It is hoped that this will lead to improvement in targeted treatments and allow patient stratification by development of novel biomarkers as discussed in the next sections.

1.4.4 Pathophysiology: VASP phosphorylation and podocyte cytoskeleton re-organisation

If circulating factors are a cause of NS (MCD or FSGS), based on histological changes discussed earlier, the main effects appear to be on podocyte structure and, in particular, the actin cytoskeleton. Mechanisms by which the factors may have this effect have been studied by several groups including at the University of Bristol. Using a conditionally-immortalised human podocyte cell line (ci-hPod) [187], Harris *et al*. showed that vasodilator stimulated phosphoprotein (VASP) is phosphorylated consistently and cell migration increased in response to treatment with FSGS relapse plasma compared with the plasma from the same patients in remission [188]. The effect appeared specific, not being observed when using plasma exchange samples from patients with other non-kidney diseases. The effect was blocked in the presence of inhibitors of plasma proteases. Protease activated receptors (PARs), which respond to proteases, were previously known to activate the MAPK and NFκB pathways and signal to the actin cytoskeleton. The group demonstrated that podocytes express PAR1-3 and treatment of ci-hPod with synthetic agonists of PAR1 and PAR2 led to VASP phosphorylation [188]. Knockdown of PAR1 but not PAR2 significantly reduced the effect of relapse plasma on VASP phosphorylation. Podocytes with a mutated form of podocin

(R138Q which is incorrectly localised) were also treated with FSGS relapse plasma but this did not lead to VASP phosphorylation [188]. This suggests that signalling via PAR-1 to VASP is dependent on correct localisation of podocin. The proposed mechanism by which a putative circulating protease leads to podocyte actin reorganisation is summarised in Figure 1.5.

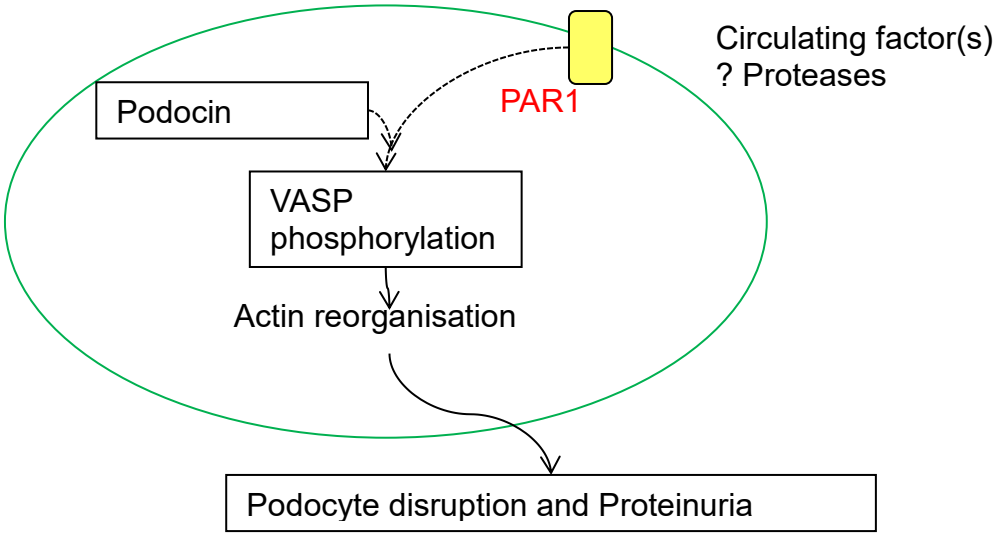


Figure 1.5: Potential mechanism by which circulating proteases lead to podocyte disruption and proteinuria

1.5 Treatments for Nephrotic Syndrome

The management of nephrotic syndrome is multi-faceted: acutely involving the control of blood pressure, fluid balance and infection; chronically, when associated with CKD, involving renal replacement therapy, bone disease and anaemia. These aspects will not be discussed further and have recently been reviewed [189]. The main focus here is on disease-modifying treatments particularly immunosuppressives and anti-proteinuric drugs.

The first-line treatment of nephrotic syndrome is steroids (glucocorticoids) namely prednisolone/prednisone or methylprednisolone. Overall, 80-90% of children respond within the first 4 weeks of treatment. In children who do not respond, and therefore classified as having SRNS, the KDIGO group published treatment recommendations in 2012 which are summarised in Table 1.7 [34].

Table 1.7: Recommendations for treatment of SRNS in children

Recommendation / Suggestions	Grade of evidence
1. Use a CNI as initial therapy for children with SRNS	1B
1.1 Continue CNI therapy for a minimum of 6 months and then stop if a partial or complete remission of proteinuria is not achieved	2C
1.2 Continue CNI therapy for a minimum of 12 months when at least a partial remission is achieved by 6 months	2C
1.3 Combine low-dose corticosteroid therapy with CNI	2D
2. Treat children with SRNS with ACEi or ARB	1B
3. In children who fail to achieve complete or partial remission on CNIs and steroids:	
3.1 Consider high-dose corticosteroids, MMF or a combination of these	2D
3.2 Suggest not giving cyclophosphamide	2B
4. In patients with relapse of NS after complete remission, restart therapy using any of the following:	2C
Oral corticosteroids	2D
Return to previous successful immunosuppressive agent	2D
Alternative immunosuppressive agent to minimise potential cumulative toxicity	2D

Legend: ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CNI, calcineurin inhibitor; MMF, mycophenolate mofetil. Grade of evidence: 1, "we recommend"; 2, "we suggest"; B, moderate quality of evidence; C, low quality of evidence; D, very low quality of evidence [190].

One study has compared the CNIs ciclosporin and tacrolimus directly which showed no significant difference in the control of proteinuria (80% vs 85.7% respectively) and similar rates of side effects including hypertension, nephrotoxicity and diabetes mellitus [191]. Gum hyperplasia and hypertrichosis were more frequent in patients on ciclosporin. Proteinuria generally decreased

within 4 weeks with median times to remission (either complete or partial) in the RCT being 8 and 12 weeks for tacrolimus and ciclosporin respectively.

A Cochrane review from 2016 provides the most comprehensive analysis of treatments available for SRNS [192]. Studies included were RCTs of children 3 months to 18 years with SRNS comparing immunosuppressive or non-immunosuppressive treatments with each other, with placebo or corticosteroids. Nineteen, mostly small, RCTs with 773 evaluable children were included and of these 9 were published before 2000. Overall, CNIs were associated with a greater rate of complete and partial remission compared with no treatment/placebo or cyclophosphamide. One study (41 children) found no significant difference in complete remission between ciclosporin and tacrolimus. The use of ACEi was found to reduce proteinuria significantly in two studies. One of the 19 studies in the Cochrane review excluded patients if mutations had been detected in *NPHS2* or *WT1* [193], one excluded patients with hereditary or syndromic disease [194], and in one study all patients had genetic testing for *NPHS2* and *WT1* exons 8 and 9 but this was not used this to determine recruitment [191]. Although the criterion of being over 3 months likely excluded some patients with genetic disease, it is probable that most studies included a mixture of patients with genetic and non-genetic SRNS.

The failure of many therapeutic studies in FSGS/SRNS to phenotype patients adequately and only include a relatively homogeneous group, or at least stratify patients with adequate numbers in each subgroup to allow analysis, has been identified as a reason for inconclusive outcomes [195, 196]. The use of multiple parameters, such as genotyping, pattern of steroid resistance (primary/presumed vs secondary steroid resistance) and age of disease onset to

stratify patients with SRNS are examined in Chapters 2 and 3 and these are likely to be important in future clinical trials.

As illustrated by the levels of evidence in Table 1.7 (mostly grade C or D), other than initial treatment with CNIs and ACEi/ARBs, subsequent management of SRNS has not be widely subjected to randomised controlled trials. Most evidence derives from smaller, non-randomised cohort studies which, until recently, did not have comprehensive genotyping data to include in the analysis. The range of treatments used for NS together with related evidence is summarised in Table 1.8.

Table 1.8: Disease-modifying treatments in SRNS

Medication	Mechanism of action	Indication	Typical dose and route	Evidence / Clinical trials	Key references
Prednisolone	Reduces T and B cell proliferation, supresses antibody production, blocks IL-2	New presentation of NS Relapses	60mg/m^2 or 2mg/kg daily for 4-6w then 40mg/m^2 or 1.5mg/kg alternate days		
ACEi	Renin-angiotensin system blockade	Anti-proteinuric in SRNS	Enalapril 0.2-0.6mg/kg/d	Dose-related reduction in proteinuria (52% with enalapril 0.6mg/kg). Fosinopril with prednisolone significantly reduces proteinuria compared with prednisolone alone	[197, 198]
ARB	Renin-angiotensin system blockade	Anti-proteinuric in SRNS	Losartan 0.7mg/kg/d	RCT in adults with FSGS: significant decrease in proteinuria and increase in plasma albumin at 6 and 12m in losartan vs untreated control group	[199]
Ciclosporin	Calcineurin inhibitor; blocks T-cell proliferation	SDNS SRNS	2-3mg/kg bd po	Three RCTs in SRNS: n=49. CR 31%, PR 38% within 6m vs 0-16% in control groups	[34]
Tacrolimus	Calcineurin inhibitor; blocks T-cell proliferation	SRNS	0.1-0.2mg/kg/d po in 2 divided doses	Tacrolimus vs Ciclosporin: no significant difference in control of proteinuria	[191]
Mycophenolate mofetil	Inhibitor of inosine-5-monophosphate dehydrogenase	SDNS FR-SSNS SRNS	600mg/m^2 bd po	CR or PR: 65% in SSNS, 67% in SRNS CR or PR: 33% in MMF + oral dexamethasone arm of RCT (no difference from ciclosporin)	[200, 201]

Cyclophosphamide	Cytotoxic alkylating agent, depletion of immune cells	SDNS FR-SSNS Not recommended in SRNS	2mg/kg/d po for 12w 500mg/m^2 IV monthly for 6m	Relapsing SSNS: decreased incidence of relapse at 6-12m vs prednisolone alone. FSGS: No evidence of benefit over pred alone. CR or PR: 50% vs 57%	[202, 203]
Levamisole	Alters balance between type I and II cytokines. Direct effect via induction of glucocorticoid receptors on podocytes [204]	SDNS FR-SSNS	2.5mg/kg po alternate days	Significantly fewer relapses vs placebo, pred or no treatment	[203]
Azathioprine	Purine nucleoside analogue, inhibits DNA replication	Not recommended in SRNS		RCT in SRNS: No difference in CR or PR for azathioprine + pred vs placebo + pred	[205]
Rituximab	Anti-CD20, Chimeric (mouse-human) mAb	SDNS Not recommended in SRNS	375mg/m^2 IV, 2 doses 2 weeks apart	SDNS & FR-SSNS: RCT rituximab vs placebo showed significantly longer relapse-free period (267d vs 101d) SRNS: RCT showed no additional benefit of rituximab + ciclosporin + pred vs ciclosporin + pred. Smaller non-controlled studies suggested some benefit in SRNS.	[193, 206, 207]
Ofatumumab	Anti-CD20, fully human mAb	SRNS Post-transplant recurrent FSGS	300mg/1.73m^2 IV, then 2000mg/1.73m^2 IV 6 weekly for 5 doses	CR or PR in 13/16 patients in 4 studies	[208, 209]

Abatacept	CTLA4-Ig fully human fusion protein, inhibits T cell co-stimulation via CD80	Not recommended in SRNS	10mg/kg IV repeated 2 weeks later	CR or PR in 5 patients with FSGS and $CD80^+$ podocytes on renal biopsy. No improvement in 5 patients with post-transplant FSGS recurrence	[210, 211]
Adalimumab	Anti-TNF, Fully human mAb	Not recommended in SRNS	24mg/m^2 sc every 2 weeks	More than 50% reduction in proteinuria in 4/10 patients with SRNS in phase I study RCT: no patients in adalimumab arm (n=7) reached primary outcome	[169, 179]
Galactose	Inhibits putative circulating factor	Possible adjunct in SRNS	0.2g/kg bd	Decrease in circulating factor activity but no effect on proteinuria	[170]

Summarised from [34, 192, 203, 212]. Legend: ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; bd, twice daily; CR, complete remission; d, days; IV, intravenous; m, month; mAb, monoclonal antibody; MMF, mycophenolate mofetil; po, by mouth; PR, partial remission; pred, prednisolone; RCT, randomised controlled trial; sc, subcutaneous; TNF, tumour necrosis factor; w, weeks

1.5.1 Non-immunologic mechanisms of immunosuppressants in SRNS

Many of the treatments used for SRNS are immunosuppressants which accords with our understanding that non-genetic disease involves an immune-mediated circulating factor. With greater understanding of the pathogenesis of NS, several drugs are now thought to have direct actions on podocytes [213]. The first-line treatment, glucocorticoids, have widespread effects on the immune system but it has been recognised that they can stabilise the actin cytoskeleton and promote survival in podocytes [149, 214].

Ciclosporin does have an immunosuppressive effect via inhibiting the activity of calcineurin and so nuclear factor of activated T cells (NFAT). This prevents transcription of IL-2 and other cytokines and reduces activation of T cells. This was thought to be its primary action in SRNS. However, more recent studies have suggested a direct effect on the podocyte cytoskeleton by blocking the calcineurin-dependent dephosphorylation of synaptopodin [215]. If one mechanism of action is directly on stabilising the actin cytoskeleton, this may explain why ciclosporin does appear to have some effect in genetic forms of FSGS which is presumed to be non-immune mediated. In one study, ciclosporin led to remission in 78% of patients with non-genetic disease compared with 19% of those with genetic disease [138].

TNF inhibitors, including infliximab, adalimumab and etanercept, have been tried in patients with recurrent FSGS with some limited effect [178, 179]. *In vitro* studies using ci-hPod have shown that the cytoskeletal disruption caused by exposure to plasma from patients with relapsed FSGS was blocked in a dose-dependent manner by anti-TNF antibodies [175]. By treatment of podocytes with

TNFα they found evidence that it activates β3 integrin which *in vivo* could impact on cell adhesion to the GBM.

Rituximab is a chimeric mouse-human monoclonal antibody directed against CD20 which is expressed on B cells. One mechanism of action is by reduction in B cell numbers so reducing autoantibody production and antigen presentation/co-stimulation of T cells. In SRNS, another potential mechanism is by binding to sphingomyelin phosphodiesterase acid-like 3b (SMPDL-3b) on podocytes which promotes stabilisation of the actin cytoskeleton [216].

Abatacept is a CTLA-4 agonist and CD80 inhibitor which has its immune system effects by blocking the co-stimulation of T lymphocytes by CD28. Podocytes have been shown to express CD80 and it seems to have a role in actin cytoskeleton regulation. In mouse and *in vitro* podocyte models, treatment with lipopolysaccharide (LPS) led to increased expression of CD80 in podocytes, actin reorganisation, effacement and nephrotic syndrome [217]. The effects were not seen in $CD80^{-/-}$ mice. In a cohort of 5 patients with CD80-positive podocytes confirmed on renal biopsy, treatment with abatacept resulted in sustained remission of NS in all cases [210]. However, this response has not been confirmed in subsequent larger studies [218, 219].

Taken together, knowledge of the direct effects of immunosuppressive drugs and improvements in understanding of SRNS pathogenesis highlight avenues for exploration in development of novel therapies targeting podocyte signalling and the actin cytoskeleton. It will be important to test the drugs in well-phenotyped and biologically-characterised patients.

1.5.2 Plasmapheresis

As discussed previously, post-transplant recurrence of proteinuria occurs in 30-50% of cases. Risk factors for this have been identified in a retrospective study [220]:

- A short time period between disease onset and renal biopsy
- Higher proteinuria at diagnosis
- Lower serum albumin at diagnosis
- Lower eGFR at diagnosis in relation to circulating factor disease

Plasmapheresis, which is the procedure of separating plasma from blood and retaining it outside the body, has been used as a therapy for management of post-transplant recurrence of NS. In the management of SRNS, usually large volumes of plasma are removed (1-1.5 plasma volumes / several litres) which is replaced with an albumin-containing fluid: this is termed plasma exchange (PEx) [221]. Blood is removed from a patient and, in the commonest method, subject to centrifugation which separates components by density. Plasma moves closest to the axis of rotation while red blood cells are furthest away with white blood cells and platelets intermediate. A single exchange session of 1-1.5 plasma volumes removes 60-70% of the circulating substances in the plasma. This includes clotting factors, antibodies, medications and, presumably, the putative permeability factor(s) responsible for some cases of SRNS. Complications of PEx are not infrequent (4-36%): some relate to central venous access, such as infections and pneumothorax; others due to large fluid shifts and loss of plasma proteins, such as hypotension and clotting problems [221].

In post-transplant recurrence of SRNS, a protocol of 10 PEx sessions over 2 weeks followed by weekly sessions for 2 months has been described [222]. In

some cases PEx has been used alongside cyclophosphamide [142]. Adsorption of plasma proteins (mainly immunoglobulins) onto a protein A column as an alternative to PEx has been reported in a small number of cases but the effects on reducing proteinuria appeared temporary [142, 223]. A review, published in 2010, of 12 studies reported that 49/70 (70%) children with post-transplant recurrence of FSGS and treated with PEx or immunoadsorption had partial or complete remission of proteinuria [224]. The effect was greatest with frequent PEx started early after recurrence. However, the studies were generally small, without a comparator group and with variable long-term outcomes.

There are reports of use of PEx pre-transplant and immediately post-transplant in attempts to prevent post-transplant recurrence. A report of 10 patients at high risk of recurrence, who had 8 peri-operative PEx sessions, found 70% were recurrence-free at follow-up (238-1258 days) [145]. Five patients received kidneys from living donors with PEx starting 1 week prior to transplant, and the other five from deceased donors with the first PEx within 24 hours of transplant. Other studies of prophylactic PEx have reported variable recurrence rates ranging from 0% in recipients of living donor grafts who had > 5 sessions of PEx pre-transplant, to 100% in deceased-donor recipients with a single pre-transplant session [142, 225].

1.5.3 Treatment outcomes in cohort studies of SRNS

In efforts to understand particularly the genetics of SRNS, national and international cohorts of patient with this rare disease have been established in recent years and were summarised earlier in Table 1.5. Although the main focus was genotyping, in some cases treatments and responses were recorded. These

data have mostly been published since the start of this research study in 2014. In several reports, treatment information was provided only for some of the patients with a genetic cause for disease [57, 59]. However, data from a German cohort and from the PodoNet registry allow analysis of responses to treatment in SRNS patients with and without pathogenic variants [44, 138, 141, 226].

Responses to ciclosporin were reported in a cohort of 231 patients (< 20 years at onset) with CNS or SRNS recruited at 6 centres in Germany.

Complete remission (CR) was defined as:

- proteinuria < 4 mg/m^2/hour OR
- urine protein to creatinine ratio (uPCR) < 30 mg/mmol OR
- trace of protein on dipstick analysis AND
- normalisation of serum albumin (> 3.5 g/dl).

Partial remission (PR) was defined as:

- proteinuria 4-40 mg/m^2/hour AND
- normalisation of serum albumin (> 3.5 g/dl).

The results are shown in Table 1.9 stratified by genetic versus non-genetic cause and CNS versus SRNS (onset > 3 months).

Table 1.9: Response to treatment with ciclosporin in a German cohort study of patients with genetic and non-genetic CNS or SRNS

	Non-genetic (n = 100)		Genetic (n = 131)	
Number of patients	CNS (n = 2)	SRNS (n = 98)	CNS (n = 60)	SRNS (n = 71)
Treated with ciclosporin, n / n_{info} (%)	0	82/96 (85.4)	9/56 (16.1)	32/68 (47.1)
With response data	-	81	9	32
CR (%)	-	49 (60.5)	1 (11.1)	1 (3.1)
PR (%)	-	15 (18.5)	0 (0)	5 (15.6)
NR (%)	-	17 (21.0)	8 (88.9)	26 (81.3)

Legend: CR, complete remission; n_{info}, number of patients with available information; NR, no response; PR, partial remission
Data abstracted from: Büscher *et al*. (2016) [138]

The use of ciclosporin was much higher in the non-genetic compared with genetic SRNS patients in this cohort. The timing of genetic testing and availability of results in relation to disease onset was not reported so it is unclear whether clinicians had genetic information when deciding on ciclosporin treatment or whether based purely on clinical assessment. There was some response (CR or PR) to ciclosporin in 79% of patients with non-genetic SRNS compared with 18.7% in genetic SRNS.

The PodoNet registry included patients with CNS, primary SRNS and persistent subnephrotic proteinuria of likely genetic cause with age of onset ≤ 20 years [226]. In the analysis of long-term outcomes and responses to treatments (published in 2017), patients with CNS and those lacking outcome data were excluded giving a cohort of 1354 children, of whom 1064 had genetic testing (Sanger sequencing of single genes in 607 children and next generation sequencing using a 30-gene panel in 457). CR was defined as:

- proteinuria reduction to < 100 mg/m^2/24-hour protein excretion OR

- < 0.2 mg/mg uPCR in spot urine (if age < 2 years old: < 0.5 mg/mg) OR
- a negative dipstick reading OR
- serum albumin > 30 g/L combined with dipstick trace.

PR was defined as:

- persistent non-nephrotic–range proteinuria with a 24-hour protein excretion 100-1000 mg/m^2/day OR
- uPCR 0.2–2 mg/mg (if age < 2 years old: 0.5–2 mg/mg) OR
- dipstick 1+ in combination with serum albumin > 30 g/L OR
- dipstick trace in combination with serum albumin < 30 g/L.

The response to immunosuppressive treatment during the first year after diagnosis stratified by sporadic, familial or genetic disease is shown in Table 1.10.

Table 1.10: Response to immunosuppressive treatment within the first year after disease onset in the PodoNet cohort of patients with SRNS

	Sporadic, no mutation identified	Sporadic, no genetic testing	Familial, no mutation identified	Genetic
Number of patients	713	290	139	212
Number with first year treatment response available	387	115	36	74
CR (%)	92 (23.8)	45 (39.1)	11 (30.6)	2 (2.7)
PR (%)	67 (17.3)	20 (17.4)	6 (16.7)	8 (10.8)
NR (%)	228 (58.9)	50 (43.5)	19 (52.8)	64 (86.5)

(n = 1354 total, 612 with treatment response data)
Legend: CR, complete remission; NR, no response; PR, partial remission
Data abstracted from Trautmann *et al*. (2017) [226]

Taking the two studies together, in patients with confirmed genetic cause for SRNS, there was no response to ciclosporin or other immunosuppression in over 80% of cases. Conversely, there were a few patients with genetic disease who did show CR or PR and, if these could be identified early after diagnosis, it

may be worthwhile pursuing medical treatments in these cases. In the PodoNet cohort, of 423 patients with non-genetic disease and available treatment responses, 176 (41.6%) showed CR or PR in the first year. This seems lower than the 79% combined CR or PR to ciclosporin in the German cohort, however this was not limited to the first year after disease onset. The PodoNet cohort study did provide response rates to specific therapies as show in Table 1.11.

Table 1.11: Responses to specific immunosuppressive drugs treatment episodes within the first year after disease onset in the PodoNet cohort of patients with SRNS

Treatment	CR	PR	NR	Total
CNI	129 (29.8)	82 (18.9)	222 (51.3)	433
CPH	9 (9.2)	8 (8.2)	81 (82.7)	98
MMF	2 (8.3)	2 (8.3)	20 (83.3)	24
CNI + MMF	4 (11.8)	10 (29.4)	20 (58.8)	34
IV Steroid	16 (6.8)	25 (10.6)	195 (82.6)	236
IV Steroid + CNI	4 (8.2)	5 (10.2)	40 (81.6)	49
IV Steroid + other	1 (5.9)	1 (5.9)	15 (88.2)	17
IV CPH ± other	1 (12.5)	1 (12.5)	6 (75.0)	8
RTX ± other	2 (28.6)	0 (0)	5 (71.4)	7
All 1st year treatments	168 (18.5)	134 (14.8)	604 (66.7)	906
Best response in treated patients*	150 (24.5)	101 (16.5)	361 (59.0)	612

n = 612 patients
* In patients who received more than one immunosuppressive drug in the first year, this reported the outcome of the most efficacious treatment
Legend: CNI, calcineurin inhibitor; CPH, cyclophosphamide; CR, complete remission; MMF, mycophenolate mofetil; NR, no response; PR, partial remission; RTX, rituximab
Data abstracted from Trautmann *et al*. (2017) [226]

The CNIs had the highest frequency of CR at 29.8%, followed by rituximab at 28.6%. However, the latter represented only 2 patients and the data were not stratified by genetic / non-genetic disease, therefore, comparisons between the different immunosuppressive regimens were difficult. Furthermore,

in both cohorts, some patients were concomitantly treated with ACEi or ARB which potentially confounded interpretation of the response to immunosuppression.

Treatment outcomes in the UK cohort of patients with childhood-onset SRNS will be discussed in Chapter 3.

1.6 Biomarkers in Nephrotic Syndrome

1.6.1 Biomarkers and Stratified Medicine

Biomarkers have been defined as "objective indications of medical state observed from outside the patient which can be measured accurately and reproducibly" [227]. They can be used to help categorise disease subtypes (diagnostic biomarker), to guide treatment (predictive biomarker) or provide information about long-term outcomes (prognostic biomarker) [228].

Use of the combination of clinico-pathological features with molecular markers has been described as "precision medicine". This approach is most advanced in cancer medicine where genetic and molecular markers of the tumour are used as entry criteria for therapeutic trials where drugs have been designed to target specifically the key pathogenic pathway [229]. Biomarkers are also used to predict which patients are likely to suffer serious side effects from particular treatments and avoid this.

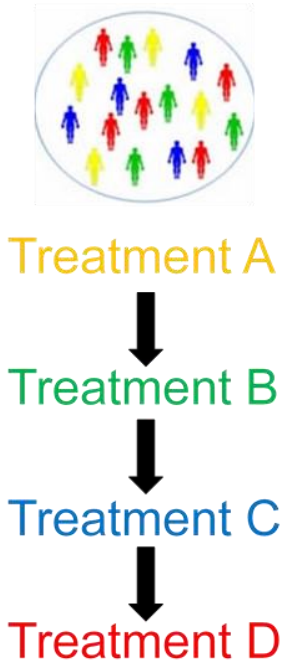
1.6.2 Biomarkers in nephrotic syndrome

Several key questions arise at first presentation or during follow-up when managing a patient with NS:

- Will the patient be steroid-sensitive or steroid-resistant?
- If steroid-resistant, what will be the most effective second-line treatment?
- Can progression to ESRF be prevented?
- In a patient with ESRF awaiting a renal transplant, will there be disease recurrence post-transplant (and how can this be prevented)?

As discussed previously, SRNS is a heterogeneous condition and better ways to stratify patients for therapeutic trials and clinical management are likely to improve outcomes and avoid giving medications to patients who are unlikely to respond but will be exposed to the side effects. The lack of clear outcomes from RCTs in NS may partly be due to the mixed groups of patients in each arm of the trial [228]. Our current and potential future strategies for management of SRNS is illustrated in Figure 1.6.

A



B

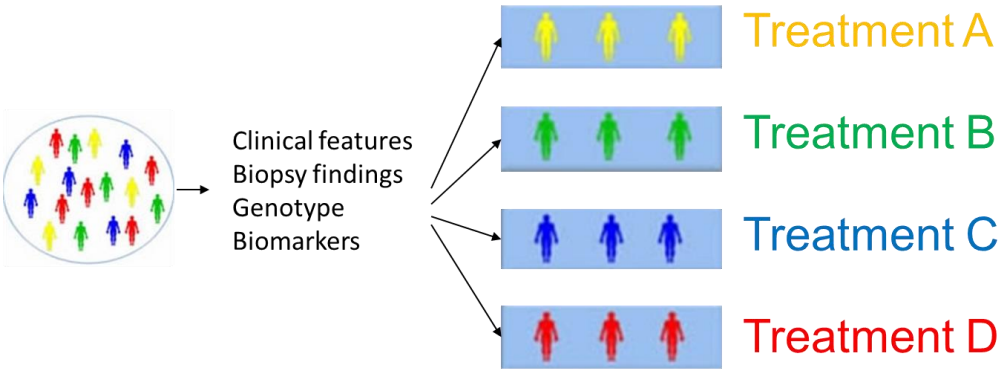


Figure 1.6: Schematic showing current and future approaches to treatment in SRNS

A: Current strategy. B. Future strategy. The colour of the figures corresponds to the treatment to which they will respond.

Using the term in the broadest sense, the biomarkers that could help stratify patients with SRNS are summarised in Table 1.12. It is likely that some of the phenotypic features (age at disease onset, family history, consanguinity) are proxies for genetic disease.

Table 1.12: Potential clinical markers and biomarkers in NS

Phenotype	**Genotype**	**Biochemical**
Sex	Genetic analysis of >50 genes associated with SRNS	Albumin at disease onset
Age at disease onset		uPCR at disease onset
Family history		Plasma or serum circulating proteins
Consanguinity		Urine proteins
Pattern of steroid sensitivity		
Renal biopsy findings		

Detailed phenotypic information and genetics have been discussed previously and will be explored in Chapters 2-4 in relation to response to treatment and long-term outcomes. Chapters 5 and 6 will examine potential plasma biomarkers and a review of the current status of circulating and urinary biomarkers is detailed below.

The potential of laboratory-measured biomarkers, either in blood or urine, have been investigated in recent years. The principles of biomarker discovery in NS are:

1. Identification of cohorts with different characteristics (e.g. SS versus SR; MCD versus FSGS; response versus non-response to a particular treatment; recurrence versus no recurrence post-transplant)
2. Collection of biological samples (usually blood or urine, with appropriate timing, processing and storage)

3. Analysis and quantification of all proteins in the samples from a "discovery cohort" of patients
4. Validation of potential biomarkers in a "validation cohort".

As discussed earlier (Section 1.2.3), renal biopsy findings do have some prognostic value but are associated with risks, particularly in smaller children. One important study examined the potential of urine biomarkers to distinguish between causes of glomerular disease [230]. Urine samples were obtained from 32 patients at the time of renal biopsy (8 with FSGS, 11 with lupus nephritis, 5 with membranous nephropathy and 8 with diabetic nephropathy). Samples from half the group were used in the discovery phase: urine was processed, and proteins separated by 2-dimensional gel electrophoresis, stained and imaged. Protein abundances were compared between groups and the combination of protein spots which indicated the cause of glomerular disease with highest sensitivity were identified. The abundance data for 21 spots were used to predict the diagnosis for the validation set, with a correct prediction in 10 out of 16 patients. Mass spectrometry (MS) of the spots identified the following proteins: albumin, α-1 antitrypsin, α-1 microglobulin, complement factor B Ba fragment, haptoglobin, hemopexin, orosomucoid, plasma retinol binding protein, transferrin, transthyretin, vitamin D binding protein and zinc α2 glycoprotein. Some of these have been identified as possible biomarkers specifically in NS as summarised in Table 1.13 below.

Several studies have sought non-invasive biomarkers, which can distinguish MCD from FSGS. The most accessible sample used in the search for novel biomarkers has been urine [231, 232]. Knowing that podocyte expression of

CD80 is increased in nephrotic syndrome, one study used enzyme-linked immunosorbent assay (ELISA) to examine urinary soluble CD80 (sCD80) levels in MCD in relapse and remission compared with FSGS, other glomerular diseases, SLE and healthy controls [233]. They found urinary sCD80 levels significantly higher in MCD relapse than remission or other conditions, but no difference in serum sCD80 levels. A similar increased urinary CD80 concentration in active MCD was reported by another group [234]. The authors suggested that urinary sCD80 may be a useful biomarker to distinguish MCD (in relapse) from FSGS. However, no large-scale clinical studies to validate this, or examine whether it could avoid renal biopsy, have been published.

Some of the possible biomarkers in NS may be directly involved in pathogenesis, such as circulating factors discussed previously (suPAR, hemopexin, IL-13, anti-CD40 antibodies) [228, 231, 232, 235]. In other cases, particularly when potential biomarkers have been discovered using proteomic techniques, there may be no clear role of the protein in pathophysiology. These more recent studies, which have taken an exploratory approach to identify proteins, metabolites and micro RNAs, are summarised in Table 1.13. Proteomics methods and analysis will be discussed in more detail in Chapter 5.

Table 1.13: Recent biomarker discovery studies in nephrotic syndrome

Aims / Target groups	Number of patients in subgroups	Samples	Methodology	Key findings	Publication (first author, year, reference)
MCD vs FSGS	Discovery: 10 MCD, 11 FSGS Validation: 14 MCD, 14 FSGS	Urine	Proteomic (2D-PAGE, MS) Validation: ELISA	AAT, TF, HTN-3, MRPL17 significantly higher in MCD. Decision tree using CALB2, MRPL17 and HTN-3 distinguished MCD and FSGS.	Pérez 2017 [236]
MCD vs FSGS	Discovery: 4 MCD, 4 MN, 4 FSGS, 4 HC Validation: 13 MCD, 26 MN, 5 FSGS, 9 IgAN, 8 HC	Urine	Proteomic (MS) Validation: ELISA	C9, CD14, SERPINA1 distinguished MCD from MN and FSGS	Choi 2017 [237]
MCD vs FSGS	10 MCD, 8 FSGS, 10 controls	Urine	Lipidomic (MS)	Increased urinary FA and LPC and decreased PC in FSGS compared with MCD	Erkan 2016 [238]
MCD vs FSGS	5 MCD (clinically defined), 8 FSGS (biopsy)	Urine (exosomes)	Micro RNA analysis (miR-193a)	Exosome miR-193a significantly higher in FSGS	Huang 2017 [239]
MCD vs FSGS	5 MCD, 16 FSGS, 5 controls	Urine, plasma (exosomes)	Micro RNA array Validation: qRT-PCR	Several plasma and urine miRs significantly increased and decreased in MCD compared with FSGS	Ramezani 2015 [240]
MCD vs FSGS vs MGN	Discovery: 12 MCD, 12 FSGS, 12 MGN, 12 HC Validation: 18 each MCD, FSGS, MGN	Urine	Metabolomic (MS)	Multi-metabolite panels are able to distinguish MCD, FSGS and MGN from each other	Lee 2016 [241]
MCD vs FSGS vs MsPGN vs MN	10 MCD, 10 FSGS, 10 MsPGN, 10 MN, 16 controls	Serum	Micro RNA expression using qRT-PCR for miR-181a, miR-483-5p, miR-557	miR-181a significantly higher in all disease groups compared with controls. No significant difference between disease groups.	Sui 2014 [242]

MCD vs FSGS vs MsPGN and others	8 MCD, 4 FSGS, 22 MsPGN and others (total n = 52)	Renal biopsy	Micro RNA expression using qRT-PCR for miR-191, miR-151-3p, miR-150, miR-30a-5p, miR-19b	miR-191 significantly higher and miR-151-3p lower in all disease samples compared to controls. miR-150 significantly different between MCD and some other subtypes	Lu 2015 [243]
FSGS vs controls	11 FSGS, 6 IgAN, 8 controls	Urine	Proteomic (MS)	77 proteins different between FSGS and healthy controls, most significantly CD59, CD44, IBP7, Robo4, DPEP1	Nafar 2014 [244]
FSGS vs MN	25 FSGS, 23 MN	Serum	Proteomic (SDS-PAGE)	Protein of molecular weight 29 kDa found significantly more frequently in FSGS than MN	Pant 2016 [245]
SSNS vs SRNS	Discovery: 15 SSNS, 12 SRNS, 5 controls Validation: 40 SSNS, 20 SRNS, 20 controls	Urine	Proteomics (MS) Validation: ELISA	Apolipoprotein A1 differentiates SRNS from FRNS/SDNS. Alpha-2 macroglobulin, orosomucoid 2 and retinol binding protein 4 can distinguish SRNS-MCD from SRNS-FSGS	Suresh 2016 [246]
SSNS vs SRNS	6 SSNS, 4 SRNS	Urine	Proteomics (MS)	Apolipoprotein A1 most increased in SSNS compared with SRNS. Matrix remodelling-associated protein 8 most decreased in SSNS compared with SRNS.	Kalantari 2014 [247]
SSNS vs SRNS	25 SSNS, 27 SRNS, 18 controls	Urine	Urine NGAL	Urine NGAL significantly higher in SRNS than SSNS, optimal cut off 0.46 ng/mg creatinine	Nickavar 2016 [248]
SSNS vs SRNS	47 SSNS, 23 SRNS	Urine	Urinary protein bound sialic acid	UPBSA significantly higher in SRNS than SSNS, optimal cut off 2.71 μg/ml of protein	Gopal 2016 [249]
SSNS vs SRNS	28 SSNS, 24 SRNS, 5 controls	Urine	Vitamin D binding protein ELISA	Urine VDBP significantly higher in SRNS than SSNS or controls	Bennett 2016 [250]
SSNS vs SRNS	20 SSNS, 16 SRNS	Blood PBMCs	Flow cytometry of P-glycoprotein expression	P-glycoprotein expression significantly higher in SRNS	Badr 2016 [251]
SSNS vs SRNS	56 SSNS, 10 SRNS	DNA	SXR rs3842689 polymorphism analysis	Del/Del polymorphism was risk factor for steroid resistance	Turolo 2016 [252]
SSNS vs SRNS	40 SSNS, 30 SRNS, 23 controls	Urine, Blood PBMCs	PMBC TLR-3, TLR-4 and CD80 mRNA expression. Urine CD80 level	Median TLR-3, TLR-4, CD80 mRNA levels higher in active SSNS than SRNS. Urine CD80 significantly higher in MCD than FSGS.	Mishra 2017 [253]

SSNS vs SRNS	47 SSNS, 23 SDNS/SRNS	Urine	Urine protein carbonyl content (UPCC) as marker of oxidative stress / free radicals	UPCC significantly higher in SDNS/SRNS group. Threshold of 5.10 nmol/mg of protein	Gopal 2017 [254]
SSNS vs SRNS	Discovery: 5 SSNS, 5 SRNS Validation: 30 SSNS, 20 SRNS	Urine	Proteomics (MS) Validation: ELISA/ immunonephelometry	VDBP, prealbumin (transthyretin), NGAL, fetuin-A, AGP2 significantly higher in SRNS.	Bennett 2017 [255]
SSNS vs SRNS	40 SSNS, 40 SRNS, 40 controls	Serum	Nephronectin (NPNT) ELISA	NPNT significantly higher in SSNS than SRNS and controls, and significantly lower in SRNS than controls.	Watany 2018 [256]
Active NS vs remission	Discovery: 4 pairs (relapse & remission) Validation: 14 pairs (3 SRNS-MCD, 11 SSNS)	Urine, plasma	Proteomics (MS) Validation: ELISA	Plasma hemopexin significantly lower in active NS compared with remission. Urine E-cadherin significantly lower in active NS compared with remission.	Andersen 2012 [257]
Predication of response to rituximab	22 FSGS receiving rituximab	Blood, T cells		$CD154^+$ $CD4^+$ $CD3^+$ T cells < 83.3%, $IFN\gamma^+$ $CD3^+$ T cells < 2.5%, $IL\text{-}2^+$ $CD3^+$ T cells < 0.3% are good predictors of rituximab response.	Chan 2016 [258]
Predication of response to LDL apheresis	SRNS treated with apheresis: 4 responders, 3 non-responders	Dextran column proteins	Proteomics (2D-PAGE, MS) of proteins from apheresis column ELISA on serum	Among column-bound proteins and in serum, SAP and APOE significantly higher in non-responders.	Kuribayashi-Okuma 2016 [259]
Prognosis of FSGS	10 FSGS, 5 with eGFR < 60 and 5 with eGFR > 60 ml/min/1.73m^2	Urine	Proteomics (MS)	54 proteins significantly increased or decreased in patients with lower GFR compared with higher GFR.	Kalantari 2014 [260]
Post-transplant recurrence vs no recurrence	14 FSGS with post-transplant recurrence, 61 FSGS without recurrence, 30 FSGS-unrelated proteinuria, 14 familial FSGS	Urine	Proteomics (2D-PAGE, MS) Validation: ApoA-I by WB	Urine levels of ApoA-Ib increased in 92.9% patients with post-transplant recurrence vs 1.6% without recurrence, 3.3% with FSGS-unrelated proteinuria, 0% familial FSGS	Lopez-Hellin 2013 [261]

Post-transplant recurrence vs no recurrence	4 FSGS with post-transplant recurrence, 9 FSGS with no recurrence	Urine	ApoA-I WB	ApoA-I positive before recurrence in 3/4; always negative in 8/9 with no recurrence. Sensitivity of 93.3% and specificity of 90.9% to diagnose FSGS relapses	Puig-Gay 2018 [262]

Legend: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; AAT, alpha-1 antitrypsin; AGP2, alpha-1 acid glycoprotein 2 (orosomucoid 2); ApoA-Ib, apolipoprotein A-Ib; APOE, apolipoprotein E; C9, complement factor 9; CALB2, calretinin; DPEP1, Dipeptidase 1; FA, fatty acids; GFR, glomerular filtration rate; HTN-3, histatin-3; IBP7, insulin-like growth factor-binding protein 7; LPC, lysophosphatidylcholines; MRPL17, mitochondrial ribosomal protein L17; MS, mass spectrometry; MsPGN, Mesangioproliferative glomerulonephritis; NGAL, neutrophil gelatinase-associated lipocalin; PC, phosphotidylcholine; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; Robo4, roundabout homolog 4; SAP, serum amyloid P-component; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SXR, alternative name for nuclear receptor subfamily 1 group I member 2; TF, transferrin; VDBP, vitamin D binding protein; WB, Western blotting

Many recent biomarker studies in NS have focused on diagnosis, either histological (MCD or FSGS) or clinical (SSNS or SRNS). Relatively small numbers of patients have been included and validation in large prospective clinical cohorts has not yet been undertaken. One study (Andersen *et* al. 2012) examined differences in urine and plasma proteins at time of relapse and remission, mostly in patients with SSNS [257]. They found that 11 proteins were significantly ($p < 0.05$) increased and 7 were significantly decreased in plasma at time of relapse versus remission. In particular, they reported that plasma hemopexin was decreased at relapse and this MS finding was confirmed by ELISA. Chapters 5 and 6 of the current study will investigate proteins which differ in relapse and remission in patients with SRNS particularly focussing on post-transplant recurrence. This has the potential to identify biomarkers and possible circulating factors. Chapter 7 will examine the effect of treatment of ci-hPod with plasma from SRNS patients at time of relapse and remission, again as a method to identify biomarkers and for insights into pathophysiology.

1.7 Aims and Hypotheses

The overall aim of this study was to investigate the interplay of phenotype, genetics and potential biomarkers with response to treatment and long-term outcome in SRNS.

The main aims were:

To undertake deep phenotyping of a cohort of patients with SRNS and examine associations between baseline characteristics and long-term outcomes (Chapter 2)

To compare characteristics and outcomes in subgroups with and without genetic disease (Chapter 2)

To examine response to disease-modifying treatments in subgroups with and without genetic disease (Chapter 3)

To evaluate the performance of genetic testing of SRNS patients in real-world clinical practice (Chapter 4)

To identify potential novel biomarkers in patients with SRNS at times of disease relapse and remission (Chapters 5, 6 and 7)

It was hypothesised that in SRNS, specific genetic and phenotypic features would be able to stratify patients into subgroups with better response to disease-modifying treatment and long-term outcome. It was also envisaged that these features would identify patients with a poorer prognosis who would be unlikely to respond to medical management but may benefit from transplantation. It was hypothesised that certain proteins in plasma (other than albumin and immunoglobulins) would be consistently different (either increased or decreased) at time of relapse compared with remission. The protein(s) may be a marker of disease activity or may be involved with disease pathogenesis (for example a circulating factor). If plasma contains a pathogenic factor, it was hypothesised that intracellular pathways in podocytes, the target cells for disease in SRNS, would be affected differently when exposed to relapse versus remission plasma. The effect may also be a marker of disease activity and give an insight into the mechanism of disease at a cellular level.

Chapter 2 The UK SRNS Cohort

2.1 Introduction

As a rare condition, much of our deepest understanding of the causes, natural history and outcomes of SRNS has derived from studies of cohorts of patients at a national and international level. One of the prime motivators has been to identify factors at diagnosis or early during the course of disease which may help to predict longer-term outcomes and response to treatment. The latter will be the focus of Chapter 3. This part of the study will examine demographic and clinical features, together with results of genetic analysis, and the association with long-term outcomes in children with SRNS in the UK national cohort.

As discussed in Chapter 1 (Table 1.5), data from other national and international studies have been published during the course of the current research. Some, such as the large international cohort reported by Sadowski *et al*. [57] included patients with adult-onset disease. All had, as a primary focus, the genotyping of patients targeting a panel of between 8 and 28 genes previously shown to be associated with SRNS. This study took the approach of whole exome sequencing (WES) which allows the identification of pathogenic variants in all the genes known to cause SRNS but also the potential to discover novel disease-associated genes.

2.1.1 Whole exome sequencing

The human genome contains 3.1 billion DNA base pairs [263]. Approximately 1% of this is coding sequence, called the exome, and is arranged into around 20000 protein-coding genes [264]. Although likely an overestimate, it has been

thought that the exome contains 85% of all pathogenic mutations associated with Mendelian diseases [265]. The Human Genome Project completed sequencing of the first human genome in 2001 using Sanger sequencing. In this process, single-stranded DNA, up to 1000 bases in length, acts as a template. A targeted primer binds to the complementary sequence. It is extended by DNA polymerase in the presence of the four normal deoxynucleotides (dNTPs) and four chain-terminating dideoxynucleotides (ddNTPs) labelled with different fluorescent colours. Separation of the reaction products on a gel allows the base letter code to be "read" from the shortest to longest. Over the past decade, massively parallel sequencing, or "next-generation sequencing" (NGS), has allowed rapid sequencing of the whole genome (WGS) or whole exome (WES) within a matter of days and at greatly reduced cost [266]. A summary of the steps in NGS using the Illumina platform is shown in Figure 2.1.

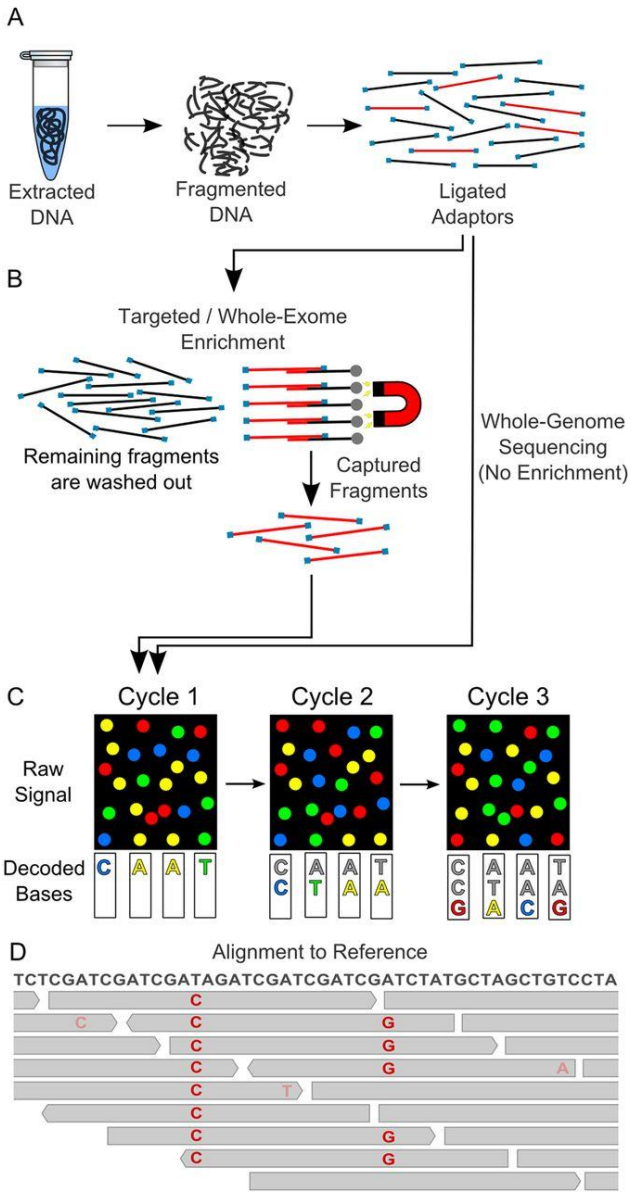


Figure 2.1: Overview of steps in next-generation sequencing

A: Patient DNA is fragmented randomly in < 1000 base pair lengths. Known adaptor sequences are ligated to fragments. B: Bait probes bind only to DNA from exons which are captured allowing intronic fragments to be washed away. C: DNA fragments bind to primers attached to a glass slide via the adaptor sequences. The fragments are clonally amplified by polymerase chain reaction to generate fragment clusters. Four fluorescently-labelled nucleotides are added to the slide and compete to be incorporated to the growing chains. In each cycle, the clusters are excited by laser and the emitted fluorescence is recorded allowing the base sequence to be "read" over time. D: Multiple overlapping DNA segments (reads) are aligned to the reference genome. The number of overlaps at a particular position is called the coverage.
Reproduced from Archives of Disease in Childhood, Schnekenberg RP & Németh AH, **99**, 284-290, 2014 with permission from BMJ Publishing Group Ltd.[267]

Sequencing of the DNA code is now relatively straightforward and the main challenge currently is in bioinformatic analysis to identify likely disease-causing variants among the vast amount of data generated [268]. In the first stage, the patient's sequence is aligned with the reference genome (Figure 2.1D) so that each nucleotide can be assigned to a particular position within a known gene. The nucleotide at each position in the patient can be compared with the nucleotide(s) seen at the same position in population databases (containing only "healthy" subjects or those known not to have the disease of interest). Such databases include the NHLBI Exome Sequencing Project (EVP), 1000 Genomes Project, dbSNP and the Exome Aggregation Consortium (ExAC). Ideally, the patient's sequence would be compared with data from a population of the same ethnicity (or genetic ancestry). However, this is more challenging for non-Caucasians who are under-represented in the population databases. Any difference between the patient's and the reference sequence is called a variant and typically 20000 – 50000 variants are identified per exome. If a variant has a clinical effect it is known as a pathogenic variant, and if it has no effect it is called a benign variant or polymorphism. If the effect is unknown, it is defined as a variant of unknown significance (VUS). The variant analysis process has been recently reviewed [269] and details of the steps used in this study are given in Methods below.

2.2 Methods

2.2.1 Patients

2.2.1.1 The National Registry of Rare Kidney Diseases

The National Registry of Rare Kidney Diseases (RaDaR) was established under the governance of the Renal Association of Great Britain following a 2008 initiative of the Medical Research Council to develop cohorts of patients with rare diseases for translational research [270]. RaDaR established an infrastructure for collection of generic and disease-specific clinical data relating to diagnosis and follow-up. The Rare Disease Group to investigate childhood SRNS was one of the first to be established. Data were collected retrospectively and prospectively via an online portal from the time of diagnosis with nephrotic syndrome and includes demographics, family history, consanguinity, pattern of steroid resistance, medications, transplantation and subsequent recurrence.

2.2.1.2 The NephroS study

The National Study of Nephrotic Syndrome (NephroS) operated within RaDaR and permited collection of biological samples including blood (EDTA) for DNA extraction and genetic testing, blood (lithium heparin) for plasma and plasma exchange effluent. Further details are available online [271]. Samples were collected with the written consent of parent(s)/guardian(s) and assent/consent of children depending on age.

2.2.1.3 Renal Patient View

Renal Patient View (PV) was a secure online portal for use by patients and clinicians which provides individuals with access to their laboratory results, letters

and medications together with generic advice about renal diseases and treatments [272]. When fully activated, the system automatically pulled laboratory results and medication data from their electronic patient records held by the NHS hospital trust or GP practice. Since 2015, for some patients at certain NHS hospital trusts, PV was directly linked to RaDaR, therefore provided a complete and current set of laboratory and medication data. For those patients without this link, data were entered manually by local clinicians and research nurses on an approximately 6-monthly basis.

2.2.1.4 Inclusion and Exclusion criteria

From its inception in 2011, the RaDaR cohort included children (< 19 years at disease onset) with SRNS. Inclusion and exclusion criteria were as follows.

Inclusion:

- < 19 years at age of onset
- Idiopathic nephrotic syndrome (nephrotic range proteinuria and hypoalbuminaemia) with no response to 4 weeks of high-dose oral prednisolone, including
    - Congenital nephrotic syndrome (presumed steroid resistance)
    - SRNS with primary steroid resistance
    - SRNS with secondary steroid resistance
    - NS as part of a syndrome, for example Nail-Patella syndrome or Denys-Drash syndrome
    - NS with FSGS on biopsy

Exclusion:

- ≥ 19 years at age of onset

- Nephrotic syndrome secondary to any other condition, including
    - IgA nephropathy
    - MPGN / C3 glomerulopathy
    - MN
    - Vasculitis
    - Systemic lupus erythematosus
    - Hypertension
    - Obesity
    - Diabetes mellitus

Following an amendment to the study protocol in December 2015, inclusion criteria were broadened to encompass patients with onset of disease at any age and all forms of idiopathic nephrotic syndrome, including SSNS, FR-SSNS and SDNS.

2.2.2 Ethical permission

Both RaDaR and NephroS (formerly the National Study of Steroid Resistant Nephrotic Syndrome in Childhood) received favourable opinions from the relevant Research Ethics Committees. For children < 16 years, parents or guardians provided written informed consent for participation with children being offered the ability to give assent. All adult patients provided written informed consent for participation. Patients/parents/guardians could choose to participate in RaDaR alone if they did not want to provide any biological samples as part of NephroS.

2.2.3 Whole exome sequencing

Anticoagulated (EDTA) blood samples were posted at ambient temperature from recruiting centres to Bristol Renal, University of Bristol. DNA was extracted at Bristol Genetics Laboratory, Southmead Hospital, Bristol, using QIAmp DNA Blood Mini Kit or Gentra Puregene Blood Kit (Qiagen, Hilden, Germany). Exome sequencing was performed in the Genomics Core Facility of the Biomedical Research Centre at Guy's and St Thomas' Hospitals and King's College, London.

2.2.4 Variant calling

Bioinformatic analysis of exome data was performed by Dr Agnieszka Bierzynska in the Bristol Renal research group. Detailed methods of variant analysis have been published in our co-authored paper [1].

2.2.5 Clinical data

Detailed phenotypic data were extracted from the RaDaR database including baseline demographics, family history, consanguinity, pattern of steroid resistance, extra-renal or syndromic features, first and subsequent biopsy findings, progression of CKD, date of ESRF, details of RRT, transplantation and post-transplant disease recurrence. These data were identified from both the relevant fields within the database and from searches of free text information copied from clinic letters. The time to ESRF and total duration of follow-up were calculated. The date of ESRF was determined as follows:

i. The date of ESRF as entered in RaDaR by the local research team;

ii. If (i) was not available, the eGFR was calculated using the Schwartz formula from the plasma creatinine and patient height. The start of ESRF was taken as the first date with eGFR persistently < 15 ml/min/1.73m^2 or the start of RRT, whichever was earlier.

The duration of follow-up was calculated between the date of diagnosis and the most recent data available in RaDaR. The most recent complete download of the database was in March 2017 and, for some patients, data continued to be entered to this point. For some patients, particularly those who moved from Paediatric to Adult care, follow-up data were available only to around age 18 years. Local research teams were approached regarding any missing data items to maximise completeness.

2.2.6 Statistical analysis

Data were analysed using Microsoft Excel 2013 (Microsoft Corporation, WA, USA) and GraphPad Prism 7 (GraphPad Software, Inc., CA, USA). Demographic and clinical features were described with frequencies and percentages and central tendency by median (inter-quartile range). The population was stratified by genetic / non-genetic status and by primary or presumed SR / secondary SR pattern. Analysis of 2 × 2 contingency tables used Fisher's exact test. Comparisons in larger contingency tables used the chi-squared test. Groups were pooled if any expected frequencies were < 5. Progression to ESRF in different subgroups was assessed using the Kaplan-Meier survival method. Analysis of differences between survival curves was by the log-rank (Mantel-Cox) test. All tests were two-tailed and $p \leq 0.05$ was considered significant.

2.3 Results

The results of this study have been previously published in a co-authored paper [1]. At the point of submission, follow-up data were available until June 2015 and of 187 included patients, 49 were identified as having genetic disease. Subsequent analysis confirmed that a further 3 patients had a genetic cause of their condition and follow-up data were available in many cases to March 2017. This chapter, therefore, presents results of the more recent analysis.

2.3.1 Cohort characteristics

The cohort included the first 187 patients recruited into the RaDaR SRNS database from inception in 2011 until 2015 who had had WES and variant analysis completed. They were recruited from 12 Paediatric Nephrology units across England, Scotland and Wales. The demographic features are shown in Table 2.1. There was an approximately-equal gender balance with almost 70% White / Caucasian and the majority (55.7%) having FSGS on their first biopsy. Data relating to family history and consanguinity were available for most patients and showed 11.8% with a positive family history and 7.2% consanguinity. Forty-five patients had some form of extra-renal features most commonly neuro-developmental delay in 16. One patient each had recognised Frasier syndrome, Pierson syndrome and Aarskog syndrome. Complete details of the whole cohort are given in Table 10.1 in the Appendices.

Table 2.1: Clinical characteristics of the cohort

		Total cohort	Patients with non-genetic disease	Patients with genetic disease	p-value
Total patients (%)		187	135 (72.2)	52 (27.8)	
Male (%)		96 (51.3)	69 (51.1)	27 (51.9)	> 0.99
Family history positive / number with data available (%)		22 / 186 (11.8)	8 / 134 (6.0)	14 / 52 (26.9)	0.0002
Consanguinity / number with data available (%)		13 / 181 (7.2)	5 / 130 (3.8)	8 / 51 (15.7)	0.0096
Ethnicity (% of patients where data available)	**White**	130 (69.5)	91 (67.4)	39 (75.0)	0.18*
	Indian	9 (4.8)	7 (5.2)	2 (3.8)	
	Black African / Caribbean	9 (4.8)	8 (5.9)	1 (1.9)	
	Pakistani	16 (8.6)	9 (6.7)	7 (13.5)	
	Bangladeshi	2 (1.1)	2 (1.5)	0 (0)	
	Asian	6 (3.2)	6 (4.4)	0 (0)	
	Mixed	10 (5.3)	9 (6.7)	1 (1.9)	
	Other	5 (2.7)	3 (2.2)	2 (3.8)	
First biopsy findings (% of patients where data available)	**FSGS**	98 (55.7)	80 (61.1)	18 (40.0)	< 0.0001†
	MCD	43 (24.4)	34 (25.9)	9 (20.0)	
	MHc	10 (5.7)	8 (6.1)	2 (4.4)	
	Finnish type	5 (2.8)	0 (0)	5 (11.1)	
	DMS	3 (1.7)	2 (1.5)	1 (2.2)	
	ESRF	3 (1.7)	0 (0)	3 (6.7)	
	Alport	1 (0.6)	0 (0)	1 (2.2)	
	TBMN	1 (0.6)	1 (0.8)	0 (0)	
	Other	12 (6.8)	6 (4.6)	6 (13.3)	
	No biopsy data available / Not biopsied	11	4	7	

Percentages are calculated for column totals. p values are for the comparison between genetic and non-genetic disease.

* For chi-squared analysis, “Bangladeshi”, “Asian”, “Mixed and “Other” were combined into one group, as shown by the square bracket

† For chi-squared analysis, “Finnish type”, “DMS” and “ESRF” were combined into one group; “Alport”, “TBMN” and “Other” were combined into another group, as shown by the square brackets

Legend: DMS, diffuse mesangial sclerosis; FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease; MHc, mesangial hypercellularity; TBMN, thin basement membrane nephropathy

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2.3.2 Age of disease onset

The age at disease onset ranged from birth (CNS) to 16 years with a median of 4.0 years (IQR 2.1-8.9 years). The distribution of patients by age group is shown in Figure 2.2. The most frequent period of onset was 1-3 years with 51 patients diagnosed at this age. The highest proportion of patients with genetic mutations was found in the 0-0.25 year group (13/15, 86.7%). When all patients were included, there was a significant association between age of onset and genetic disease ($p < 0.0001$, chi-squared, 9 df). However, there was no significant association when only patients with onset ≥ 1 year were considered ($p = 0.39$, chi-squared, 7 df).

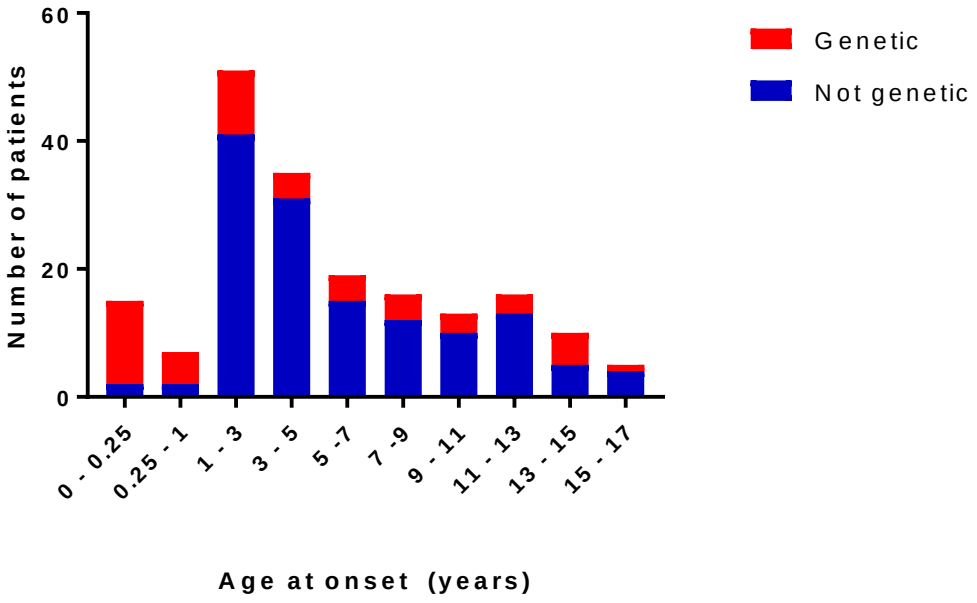

Figure 2.2: Frequency distribution of patients by age at onset of nephrotic syndrome stratified by genetic/non-genetic aetiology

2.3.3 Pattern of steroid resistance

Of 187 patients, the large majority (182, 97.3%) had SRNS. Four patients had SSNS (two with FR-SSNS) with FSGS on biopsy in three and mesangial proliferative change in one. One patient had asymptomatic persistent proteinuria with FSGS on biopsy and was not treated with steroids. The number of patients with different patterns of steroid resistance and steroid sensitivity are shown in Figure 2.3.

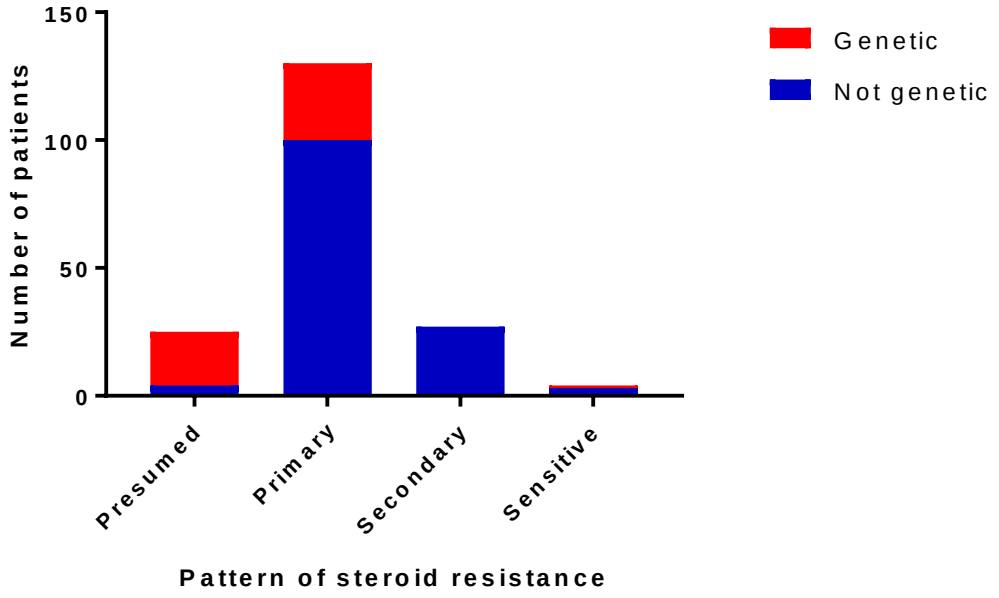

Figure 2.3: Frequency distribution of patients by pattern of steroid resistance stratified by genetic/non-genetic aetiology

Presumed steroid resistance was seen in 25 patients, of whom 16 had disease onset under 1 year of age, including all 13 with CNS. The other 9 older patients had ESRF at presentation (n = 4), a family history of genetic SRNS/FSGS (n = 4) and prominent hypertension with advanced FSGS on biopsy (n = 1). As might be expected on this basis, the frequency of genetic disease in those with

presumed steroid resistance was particularly high at 84% (21/25). Among patients with primary steroid resistance, 23.1% had genetic disease compared with none of those with secondary steroid resistance. The association of genetic disease with different patterns of steroid resistance was highly significant ($p < 0.0001$, chi-squared, 3df). One patient with FR-SSNS was found to have genetic disease and this case will be discussed in further detail in Section 2.3.7 below.

2.3.4 Incidence of genetic disease in relation to baseline clinical characteristics

A probable genetic cause was identified in 52 (27.8%) of patients (see Table 2.1 above). This did not appear to be associated with gender or ethnicity. As might be expected, there was a significantly higher frequency of a positive family history (26.9%) in the patients with genetic disease. In patients with a positive family history, the frequency of genetic disease was 63.3% (14 / 22) compared with 23.2% (38 / 164) in those with a negative family history ($p = 0.0002$, Fisher's exact test, two-sided). The cohort included two sets of siblings: two sisters with compound heterozygous *NPHS2* variants and a brother and two sisters with likely pathogenic *NUP107* variants. If families are considered rather than individuals [57], the frequency of genetic disease was 57.9% (11/19 families) in those with a positive family history.

There was a significantly higher frequency of consanguinity (15.7%) in the parents of patients with genetic disease. 61.5% (8/13) of children from consanguineous relationships had genetic disease compared with 25.6% (43/168) of children who were not from such relationships ($p = 0.0096$, Fisher's exact test, two-sided). Seven of eight children from consanguineous relationships with

genetic disease were homozygous for variants typically inherited with an autosomal recessive pattern (four children with variants in *NUP107*, two in *NPHS1*, one in *MYO1E*).

Genetic disease was significantly associated with biopsy findings when all patients were considered ($p < 0.0001$, chi-squared test with groups combined, 4 df). In all cases with a first biopsy showing CNS of the Finnish type, ESRF and Alport syndrome a genetic cause of disease was identified; however, these represent only small numbers within the cohort (9 patients in total). When considering only the most frequent biopsy findings, of 98 patients with FSGS, 18 (18.4%) had genetic disease compared with 9 (20.1%) of 43 patients with MCD ($p = 0.82$, Fisher's exact test, 2-sided). Of the 43 patients with MCD initially, 12 (32.6%) had FSGS on the most recent biopsy, 7 had persistent MCD, 2 had other findings and 22 had not been re-biopsied.

2.3.5 Long-term outcomes in patients stratified by pattern of steroid resistance

Data from RaDaR to March 2017 were used to examine long-term outcomes in the cohort. Sixty-six patients had reached the age of 18 years by this date and, for some, follow-up data were not available after transition from Paediatric to Adult care. The total follow-up time for the cohort was 1485 patient-years with a median follow-up of 4.0 years, (range 1.0-21.7 years, IQR 2.1-8.9 years).

In the whole cohort of 187 patients, 40.6% progressed to ESRF and 31.6% were transplanted (Table 2.2). Of 59 transplanted patients, 18 (30.5%) suffered disease recurrence. As would be expected, no patient who remained responsive to steroids progressed to ESRF. Among those with SRNS, patients with presumed

steroid resistance were significantly more likely to reach ESRF (76.0%) than either those with primary SR (36.9%, p = 0.0004, Fisher's exact test) or with secondary SR (33.3%, p = 0.0025, Fisher's exact test). There was no significant difference in the proportions reaching ESRF between primary and secondary SR (p = 0.83, Fisher's exact test).

Table 2.2: Long-term outcomes in the cohort stratified by pattern of steroid resistance

	Total	PSR	SSR	Presumed	SSNS	FR-SSNS	Not tried
Number of patients	187	130	27	25	2	2	1
Number (%) genetic disease	52 (27.8)	30 (23.1)	0 (0)	21 (84.0)	0 (0)	1 (50.0)	0 (0)
Number (%) who developed ESRF	76 (40.6)	48 (36.9)	9 (33.3)	19 (76.0)	0 (0)	0 (0)	0 (0)
Number (%) transplanted	59 (31.6)	39 (30.0)	6 (22.2)	14 (56.0)	-	-	-
Number (% of those transplanted) with post-transplant recurrence	18 (30.5)	14 (35.9)	4 (66.7)	0 (0)	-	-	-

Legend: FR, frequently-relapsing; PSR, primary steroid resistance; SSR, secondary steroid resistance; SSNS, steroid-sensitive nephrotic syndrome

The percentage of kidney survival over time for the different patterns of steroid resistance is shown in the Kaplan-Meier survival curves (Figure 2.4).

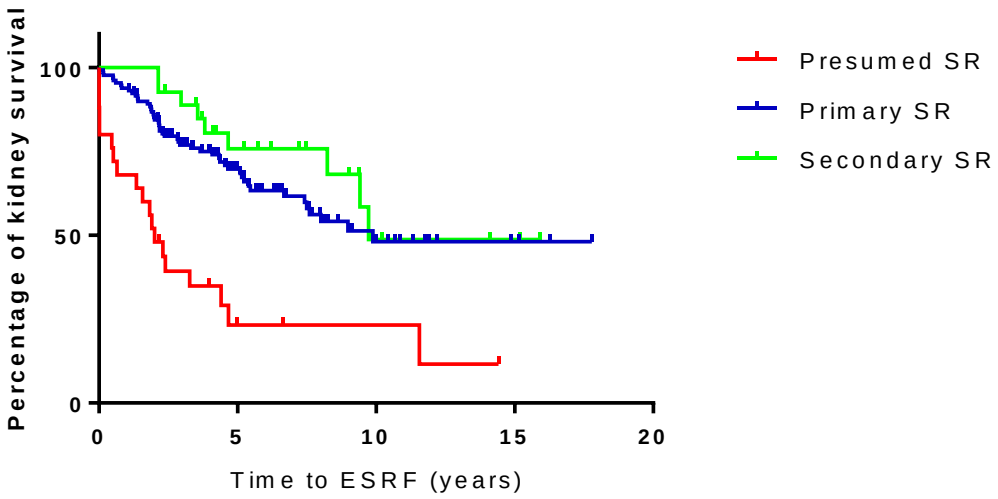

Figure 2.4: Percentage of kidney survival stratified by pattern of steroid resistance

The median time to ESRF for patients with presumed SR was 2.0 years compared with 9.9 years for patients with primary SR and 9.7 years for those with secondary SR. The differences between the curves were highly significant ($p < 0.0001$, log-rank test, 2df).

No patient with presumed SR suffered a post-transplant recurrence compared with 35.9% of those with primary SR ($p = 0.011$, Fisher's exact test) and 66.7% in those with secondary SR ($p = 0.0031$, Fisher's exact test). Although the difference between post-transplant recurrence frequency in patients with primary SR and those with secondary SR appears large, this is not statistically significant ($p = 0.20$, Fisher's exact test) most likely due to the small numbers of patients with secondary SR who were transplanted.

2.3.6 Long-term outcomes in patients stratified by genetic disease

The long-term outcomes in patients with or without genetic disease are summarised in Table 2.3 and time to ESRF in the Kaplan-Meier survival curves

(Figure 2.5). Patients with genetic disease were significantly more likely to progress to ESRF in a median of 3.3 years and none suffered post-transplant recurrence.

Table 2.3: Long-term outcomes in the cohort stratified by genetic/non-genetic aetiology

	Total	Non-genetic	Genetic	p value
Number of patients	187	135	52	
Number (%) who developed ESRF	76 (40.6)	42 (31.1)	34 (65.4)	< 0.0001
Median time to ESRF (years)	9.4	n/a*	3.3	< 0.0001
Number (%) transplanted	59 (31.6)	32 (23.7)	27 (51.9)	0.0004
Number (% of those transplanted) with post-transplant recurrence	18 (30.5)	18 (56.3)	0 (0)	< 0.0001

p values for proportions calculated using Fisher's exact test (two-sided). p value for median time to ESRF calculated by log-rank (Mantel-Cox) test of Kaplan-Meier survival curves. *The median time to ESRF for non-genetic patients is not available because less than 50% of the cohort reached ESRF. For the 42 non-genetic patients who did reach ESRF, the median time to this stage was 2.8 years.

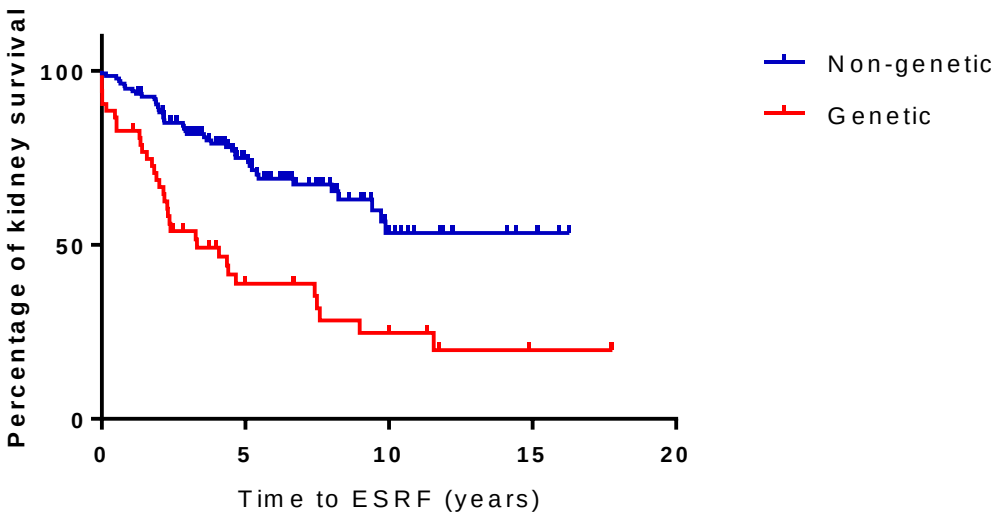

Figure 2.5: Percentage of kidney survival stratified by genetic/non-genetic aetiology

2.3.7 Genetic variants within the cohort

Whole exome sequencing identified 52 patients (27.8%) with likely-causative mutations. The genes harbouring these variants are detailed in Table 2.4 stratified by the age of disease onset. The details of the variants in 49 of the patients have been published [1]. Subsequent work by Bierzynska *et al*. confirmed novel variants in *MAGI2* to be the likely cause of disease in two patients [89] and variants in *APOL1* to be contributing to disease in a third.

Table 2.4: Frequency of likely-pathogenic genetic variants stratified by age of disease onset

Gene with likely-pathogenic variant	**Number of patients with likely-pathogenic variants by age group**					
Age group (years)	**0-0.25**	**0.25-1**	**1-5**	**5-13**	**13-17**	**Total**
Total number of patients	15	7	86	64	15	187
NPHS1	11		2	1		14
NPHS2			6	4	2	12
WT1	1		3			4
NUP107			1	2	1	4
MAGI2		2				2
TRPC6			1	1		2
LMX1B					2	2
LAMB2	1					1
COL4A5		1*				1
CRB2		1				1
MYO1E		1				1
DGKE			1			1
COL4A3				1		1
NUP93				1		1
ADCK4				1		1
APOL1				1		1
ACTN4				1		1
OCRL				1		1
PODXL					1	1
Total (%) with variants	13 (86.7)	5 (71.4)	14 (16.3)	14 (21.9)	6 (40.0)	52 (27.8)

Zeros are omitted for clarity. * This patient also had two different homozygous missense variants in *MYO1E*

NPHS1 variants were the most common cause for CNS, being responsible for 11 of 13 cases. Interestingly, one of these patients (patient 353), with onset at age 2 months, was steroid sensitive with frequent relapses and remained steroid sensitive with no progression to CKD during 17 years of follow-up. Although mostly causing early-onset disease, the oldest child (patient 414) with *NPHS1* pathogenic variants had onset at 5.7 years. Of 14 patients with *NPHS1* mutations,

11 progressed to ESRF within 0.5-4.6 years and were subsequently transplanted with no disease recurrence.

NPHS2 was the cause of presumed or primary steroid-resistant SRNS in 12 children with median age of onset of 4.9 years. Nine (75%) have progressed to ESRF within 1.3-11.6 years and, at most recent follow-up, 7 had been transplanted.

2.4 Discussion

2.4.1 Findings of this study

The data presented here from a UK cohort of 187 patients with childhood-onset SRNS showed that baseline demographic and clinical characteristics were differentially associated with genetic versus non-genetic disease. Children with disease onset under 1 year of age, with a positive family history or with consanguinity were significantly more likely to have genetic aetiology. The pattern of steroid resistance strongly predicted non-genetic disease: none of 27 patients with secondary steroid resistance was found to have a likely-pathogenic variant. In contrast, the histology findings of the first renal biopsy, excluding the 9 patients with CNS of the Finnish type, ESRF and Alport syndrome, did not help to differentiate between patients with genetic versus non-genetic disease. Approximately 20% of patients with FSGS or MCD were found to have likely-pathogenic variants.

Regarding long-term outcomes, both the presence of genetic disease and presumed steroid resistance (of which group 84% were genetic) were strong predictors of progression to ESRF but none of these patients suffered post-transplantation recurrence. In comparison, patients with secondary steroid resistance were significantly less likely to require a transplant but had a higher risk of recurrence.

A flow chart summarising patient stratification including data from this study is show in Figure 2.6.

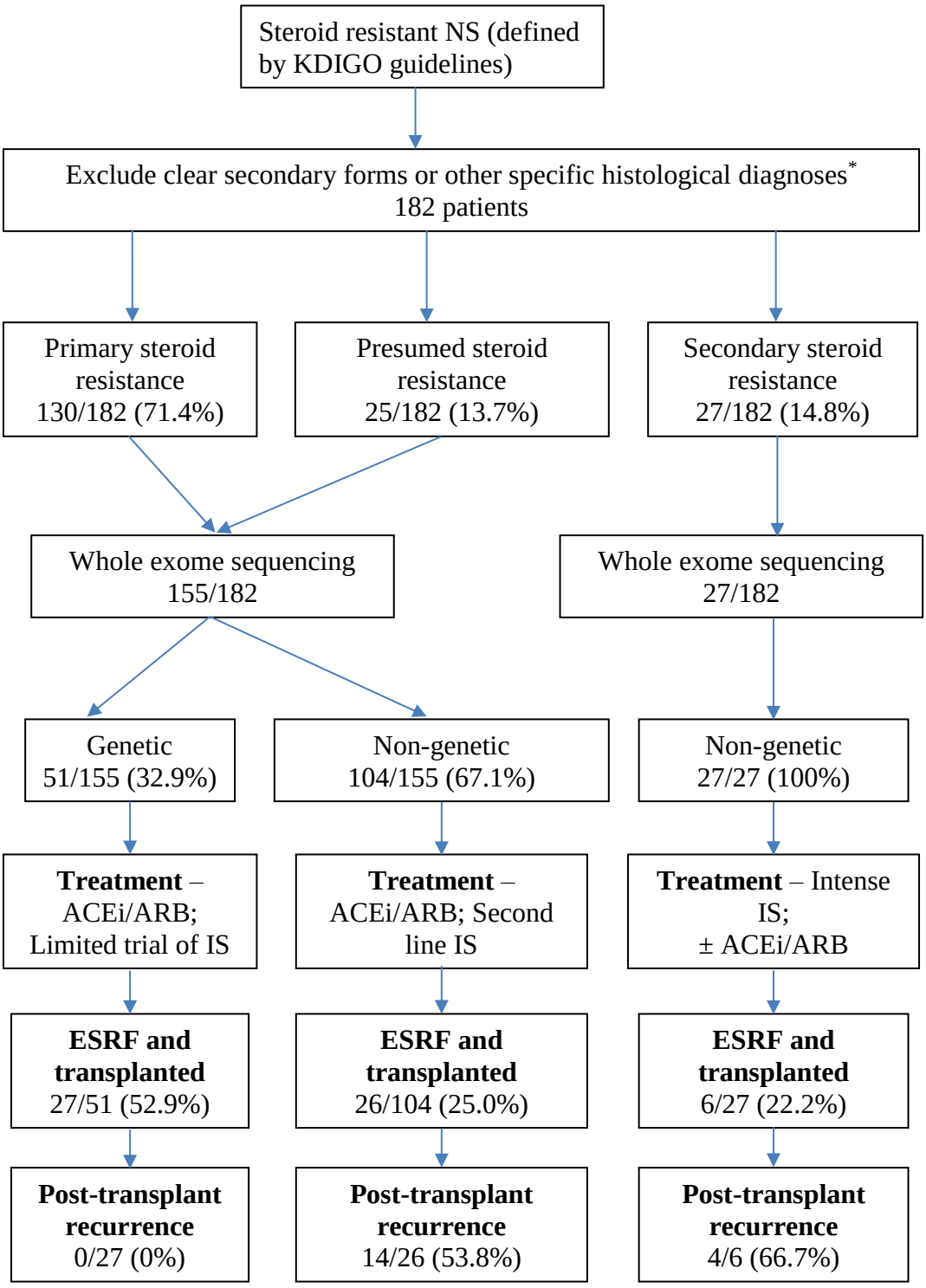

Figure 2.6: Flow chart summarising stratification of patients by pattern of steroid resistance and genetic testing

*Four patients with SSNS and one with persistent proteinuria not treated with steroids were omitted from the original 187 for this analysis.
Legend: ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; ESRF, end-stage renal failure; IS, immunosuppression; KDIGO, Kidney Disease Improving Global Outcomes

Five patients from the original cohort of 187 were omitted from the analysis. Although they met criteria for inclusion in the RaDaR study as defined previously (Section 2.2.1.4), they did not have steroid resistance. They were, therefore, excluded from Figure 2.6 so that more homogeneous groups were shown in this analysis.

The overall genetic detection rate in this cohort using WES was 27.8%, with likely-pathogenic variants found in 19 different genes. Although in some cases the clinical presentation could reasonably predict the affected gene (with 85% of children with CNS having *NPHS1* variants), some patients had variants in genes less frequently involved in childhood onset disease as previously published [1]. It is possible that not all pathogenic variants were detected by this study. Causative mutations in regulatory sequences within introns would not be sequenced by WES. Furthermore, as bioinformatic databases expand and functional studies are performed in future, variants which have been considered of unknown significance may be reclassified as likely-pathogenic.

2.4.2 Comparison with other studies

The current study was the first to use whole exome sequencing in all patients with SRNS included in the cohort. This enabled pathogenic variants to be detected not only in genes previously known to be associated with SRNS but also novel genes to be identified. As discussed previously in Chapter 1, other national and international cohort studies genotyping patients with SRNS tested panels of between 8 and 28 genes (see Table 1.5). Furthermore, in two studies including patients in the PodoNet cohort and German CNS/SRNS follow-up study, the number of genes and exactly which genes were screened varied depending on the

recruiting centre or on an algorithm based on patient age, renal histopathology and/or syndromic features [44, 138]. As discussed previously and in our co-authored paper [1], the range of clinical phenotypes associated with variants in a specific gene is broadening, therefore selecting which genes to test based on the clinical presentation risks incorrectly labelling patients as having non-genetic disease.

This study comprised patients recruited from 12 centres in a single country. While the number of patients who underwent WES and were included in analysis is felt to be large enough to draw reasonable conclusions, it is smaller than published international studies by Sadowski *et al.* (2014) which included 2016 patients and Trautmann *et al.* (2015) with 1655 patients [44, 57]. Compared with that of Trautmann *et al*., the current study had a lower frequency of a positive family history (11.8% vs. 25.6%) and consanguinity (7.2% vs. 28.6%) likely as a result of the former including patients from Turkey and the Middle East. Despite this, genetic disease was identified in 23.6% of 1174 patients tested compared with 27.8% in this study. One could speculate that this relatively higher detection rate in a population with a lower prior probability of genetic disease was the result of the WES approach. The cohort of Sadowski *et al*. reported consanguinity in 20.9% of the 1783 included families. The frequency of a positive family history was not reported. The overall rate of genetic disease was 30.5%, possibly reflecting a standardised NGS strategy for a panel of 27 genes tested in all included patients.

The studies of Sadowski *et al.* and Trautmann *et al.* did not stratify patients by pattern of steroid resistance, therefore, a direct comparison of the subgroups is not possible. A previous study from our group at the University of

Bristol together with collaborators from France examined outcomes in a cohort of 150 patients with SRNS who had been transplanted and stratified data by pattern of steroid resistance. Of 28 patients with secondary steroid resistance, 9 had genetic testing and none was found to have likely-pathogenic variants, consistent with the findings of the current study. Among the same 28 patients, the recurrence rate post-transplantation was 92.9%. In this study, among the 6 patients with secondary steroid resistance, the recurrence rate post-transplantation was 66.7%. Although this appears lower, the small numbers limit interpretation of whether this is a significant difference.

Regarding long-term outcomes, this study had a median follow-up time for the cohort of 4.0 years (IQR 2.1 -8.9 years, range 1.0-21.7 years) while the median time to ESRF was 9.4 years (based on Kaplan-Meier survival curve analysis). Overall, 5-year renal survival was 65.1%. The follow-up time was comparable to the median 3.6 years (IQR 1.5-6.8 years, maximum 15 years) reported by Trautmann *et al.*(2017) in a long-term follow-up analysis of the PodoNet cohort (n = 1354) and 5-year renal survival was 74% [226]. They, however, reported median time to ESRF of 2.8 years. Although the authors stated that they used the Kaplan-Meier analysis to determine the median time to ESRF, clearly over 50% of the cohort had not reached ESRF by 5 years. Their 10-year and 15-year renal survival rates were 58% and 48% respectively suggesting that the actual median time to ESRF was some point after 10 years and, therefore, longer than in the present study. Their analysed cohort did not include patients with CNS (onset < 3 months of age) and only 15.7% had proven genetic disease (290 of 1354 did not have genetic testing) which may explain the relatively better

outcomes in the PodoNet study. They reported outcomes stratified by response to immunosuppressive treatment and this will be a focus of Chapter 3.

2.4.3 Limitations

Recruitment of patients to the RaDaR SRNS study was conducted by Paediatric Nephrologists across Great Britain. Although any children meeting the inclusion criteria were eligible, because the study offered WES on a research basis, there is the potential for bias leading to increased recruitment of patients with suspected genetic disease or those without a diagnosis based on local clinical genetic testing. However, comparison of the overall genetic rate of 27.8% in this study with data from other cohorts (as detailed in Table 1.5) shows the figure is broadly in line at around 30%.

Clinical phenotyping data were provided by local Paediatric Nephrology research teams and inputted into the online RaDaR database. Although more recent follow-up information was collected prospectively, data relating to baseline and original diagnosis were retrospective. After initial data download, as part of this research, all centres were contacted individually with requests for specific data to maximise completeness. Despite this, information about consanguinity, ethnicity and initial biopsy findings were not available for all patients, particularly in a few cases where they were first diagnosed in a different centre or country.

Regarding long-term outcomes, data were available until the most recent RaDaR download in March 2017. Some patients who were destined to reach ESRF may not yet have reached that stage or been transplanted by that point. In addition, patients who reached the age of 18 years and transferred to Adult

Nephrology clinics were in some cases lost to follow-up. Both these factors could lower the apparent rate of progression to ESRF.

2.4.4 Conclusions

Secondary steroid resistance was associated in all cases in this study with non-genetic disease. This raises the question of whether, in clinical practice, genetic testing should be conducted only for patients with primary or presumed steroid resistance. Genetic testing in a clinical context using a National Health Service (NHS)-approved gene panel will be examined in detail in Chapter 4. Secondary steroid resistance also predicted a higher rate of post-transplant recurrence pointing to the presence of putative circulating factors. Analysis of the plasma proteome in some patients with such recurrence will be the focus of Chapter 5. This chapter has examined long-term outcomes in relation to non-modifiable, baseline patient factors (genetics, age at disease onset, pattern of steroid responsiveness and renal histology). Chapter 3 will investigate in more detail the effects of and response to immunosuppressive treatments.

Chapter 3 Response to Immunosuppression in the UK SRNS Cohort

3.1 Introduction

Chapter 2 showed that certain baseline demographic and clinical characteristics in patients with SRNS are associated with genetic disease and that both genotype and phenotype can predict, to some extent, long-term outcomes. The optimum immunosuppressive treatment strategies for patients with SRNS are not known since large, well-controlled clinical trials comparing therapies in stratified groups of patients have not been conducted [195, 196]. In the absence of prospective trials, retrospective cohort studies have been used to investigate whether baseline characteristics affect response to treatment and the effects of immunosuppressive drugs on long-term outcomes.

As discussed in Chapter 1, data from a German cohort of 231 patients with CNS or SRNS were published in 2016 [138] and from the PodoNet cohort of 1354 in 2017 [226]. Overall, a minority (< 20%) of cases with confirmed genetic cause for SRNS responded to immunosuppression compared with over 40% of patients with non-genetic disease achieving complete or partial remission on treatment (see Table 1.9 and Table 1.10).

The aim of this study was to examine the response to immunosuppressive treatment in the UK cohort of patients with childhood-onset SRNS stratified by genetics and baseline clinical characteristics.

3.2 Methods

3.2.1 Patients

The RaDaR cohort of patients with NS was used as the source of cases as described in Chapter 2. At March 2017, a total of 1785 patients had been recruited. Two groups of patients were included for analysis of response to treatment. The first group was the same 187 patients who had had genetic analysis by WES and were described in Chapter 2 and previously published [1]. In order to expand the cohort and include patients with comparable clinical features, the RaDaR cohort of 1785 was filtered by age of onset < 18 years and then by diagnosis of SRNS (Figure 3.1). The 187 patients already identified were excluded leaving 181 patients. The "gene test" sections and free text entries within the RaDaR database were searched to discover those who had had clinical genetic testing with results available. RaDaR consent permitted access to participants' medical records, therefore the results of genetic testing undertaken by Bristol Genetics Laboratory (BGL) at Southmead Hospital were checked for the 181 patients. BGL offered clinical genetic testing through the NHS using an NGS panel of 37 genes associated with SRNS. This will be the focus of Chapter 4. By these means, an additional 63 patients with genetic test results were identified providing a total of 250 for analysis.

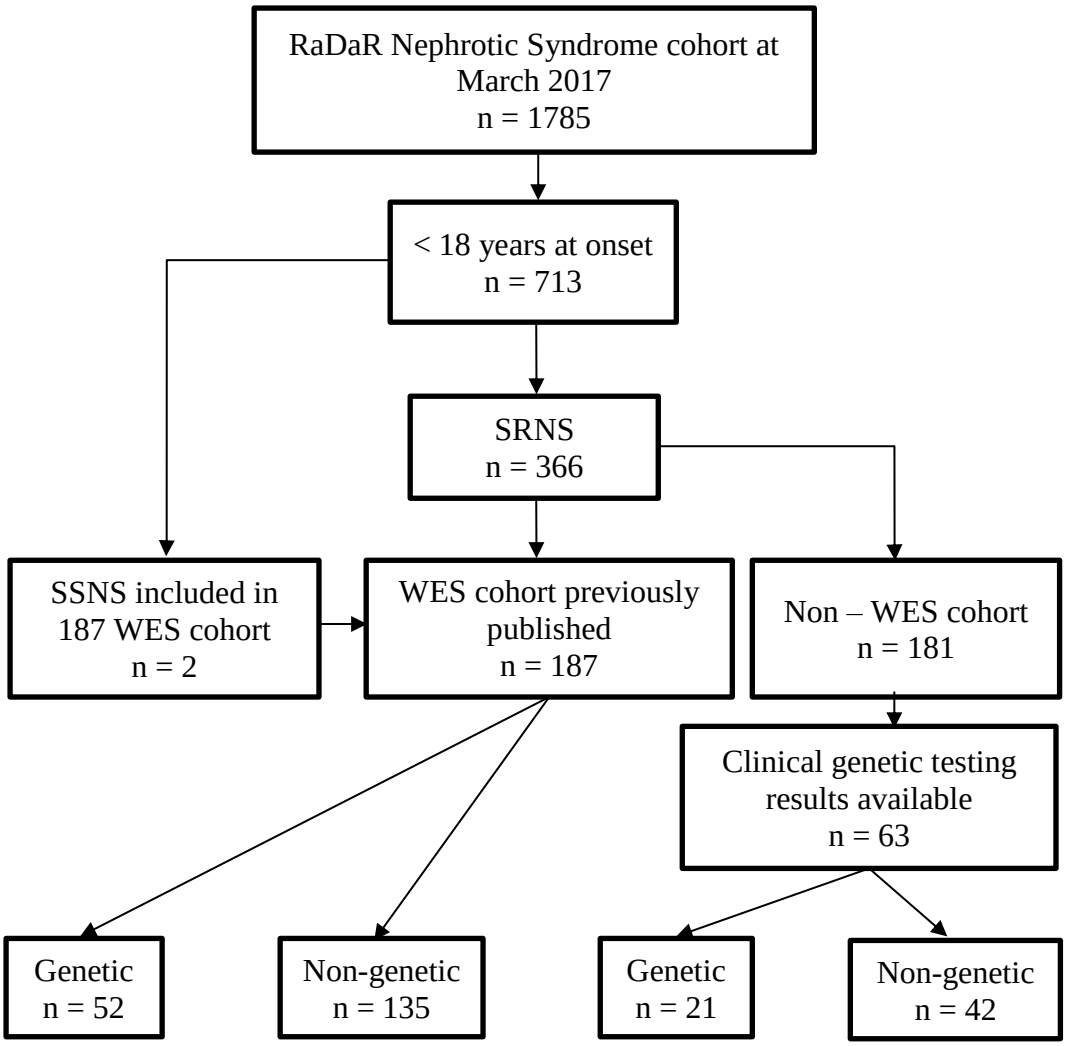

Figure 3.1: Flowchart of patients from the UK RaDaR Nephrotic Syndrome cohort showing those included in the analysis of response to immunosuppressive treatment

Legend: WES, whole exome sequencing

3.2.2 Clinical data

Demographic, clinical and long-term outcome data were extracted from the RaDaR database as previously described in Section 2.2.5.

3.2.3 Medication data

The RaDaR database current to March 2017 was downloaded into Microsoft Excel and filtered on the 250 patients included in this part of the study. The medication fields including the name of the drug, and start and stop dates, were extracted. In order to avoid missing treatments which had not been entered in the correct sections of the registry, a search was conducted on free text sections of the database which in many cases included anonymised copies of patient clinic letters. The text search terms are summarised in Table 3.1.

Table 3.1: Terms for free text search for medications

ACEi/ARB	Immunosuppressive drugs
ACEi	Azathioprine
ARB	Ciclosporin
Captopril	Cyclophosphamide
Enalapril	Levamisole
Irebsartan	Mycophenolate mofetil
Lisinopril	Rituximab
Losartan	Tacrolimus
Ramipril	
Valsartan	

The search also used the associated medication proprietary names, common abbreviations (e.g. MMF) and alternative spellings (e.g. cyclosporin).

Since the aim of this study was to examine the disease-modifying anti-proteinuric effect of the medications, they were filtered to include only those with a start date prior to the date of onset of ESRF. For each patient, the medications

were sorted by chronological order of start date. For IIS drugs, only the first course of each drug was included for analysis. Therefore, for a particular medication, each treatment course corresponded one-to-one with a different patient. This enabled valid analysis on the basis of the number of treatment episodes (which was equivalent to the number of independent patients).

Since the evaluation of response (discussed below) used a window of 6 months after the start date, in the case of rituximab this may include several intravenous doses. For ACEi and ARB, individual drugs were considered by class and only the first course within each class was included for analysis. For example, in a patient who first received enalapril and was later changed to lisinopril, only the response to enalapril would be evaluated.

3.2.4 Management of missing medication data

The list of all patients receiving at least one ACEi/ARB or IIS prior to ESRF was compared with the total list of 250 patients. The RaDaR data for those appearing not to be receiving any of these medications were reviewed in detail. In some cases, a reason for the lack of medication became evident including:

- CNS
- The patient already being in CKD or ESRF at presentation
- SRNS as part of a syndromic or familial pattern
- SSNS (who were included in the 187 WES cohort).

If no explanation became obvious, the research teams at local recruiting centres were contacted for more information.

3.2.5 Analysis of response to medication

The primary outcome was defined using the change in plasma albumin and proteinuria before and within 6 months after starting the medication.

Complete response (CR) was defined as:

- Urine protein:creatinine ratio (uPCR) < 20 mg/mmol or negative/trace dipstick proteinuria within 6 months of starting therapy.

Partial response (PR) was defined as:

- uPCR > 20 mg/mmol or dipstick ≥ 1+ but plasma albumin > 25 g/L within 6 months of starting therapy.

In cases where the plasma albumin was already > 25 g/L prior to starting treatment but remained above this in the following 6 months and proteinuria did not reach the threshold for CR, this was classed as PR.

The following laboratory data were extracted from the RaDaR database for all patients who received the medications under investigation:

- Plasma albumin
- uPCR
- Urine dipstick protein
- Urine albumin:creatinine ratio (ACR)

If all measures of proteinuria were available, preference was given to uPCR. If only urine ACR was available, a value < 3.0 mg/mmol was considered equivalent to uPCR < 20 mg/mmol.

In the cases where the RaDaR record was electronically linked to laboratory data via Renal PV, complete results were available. In some cases, however, only limited laboratory data were available which had been entered manually by the research teams. The data closest to, and prior, to the medication

start date were taken as the baseline. Where complete results were available, the lowest uPCR and highest plasma albumin achieved together were used to judge against the criteria for CR and PR. In some cases, only single results were available during the time frame.

If a medication was stopped within 6 months of starting, only laboratory data while receiving the medication were used to judge response, except in the cases of rituximab and IV cyclophosphamide which were given as intermittent doses rather than daily. If two medications were started simultaneously or within 1 month of each other, the same response outcome was assigned to both although it was not possible to determine which of the two, or the combination, was responsible for any improvement.

3.2.6 Management of missing medication response data

After completion of the above analysis, the medications for which a response could not be assigned were identified. In all cases this was due to incomplete laboratory data. Research teams at recruiting centres were approached to provide the relevant missing laboratory results. In addition, they were given an option to complete a spreadsheet listing their patients and ACEi/ARB and IIS medications which they were invited to complete to indicate which medications they had received and the response using the same criteria for CR and PR as above.

In order to maximise medication response completeness, free text entries in RaDaR in the period after the start date were reviewed both for laboratory results and the clinician opinion. If laboratory data alone, sought in a variety of ways, were insufficient to make a judgement on medication response, the overall

clinician opinion and statements such as “absence of proteinuria” or “in remission” were used to assign a response.

3.2.7 Data analysis

The proportions of patients achieving CR and PR for each medication were calculated for the cohort as a whole and stratified by genetic/non-genetic disease and by pattern of steroid resistance. As discussed in Chapter 1, patients with non-genetic disease who suffer post-transplant recurrence represent those most likely to have a pathogenic circulating factor. The response to IIS medications was examined particularly in this subgroup. Since clinicians often use IS drugs in a particular sequence, some drugs were used more often only after failure of others in patients who are then considered more “resistant”. To attempt to avoid this bias, outcomes for only the first IIS drug used per patient were analysed.

3.2.8 Statistical analysis

Data were analysed using Microsoft Excel 2013 and GraphPad Prism 7. Comparisons for proportions between cohorts and groups with data in 2 × 2 contingency tables used Fisher’s exact test. Other analysis was conducted as previously described in Chapter 2.

3.3 Results

3.3.1 Patient characteristics in the two cohorts

The demographic features of the 187 patients who underwent WES have been described in Chapter 2. The characteristics of the additional 63 patients who had clinical genetic testing (CGT) are shown in Table 3.2.

Table 3.2: Demographic characteristics of 63 patients who had clinical genetic testing

		Total cohort	Patients with non-genetic disease	Patients with genetic disease	p-value
Total patients (%)		63	42 (66.7)	21 (33.3)	
Male (%)		31 (49.2)	23 (54.8)	8 (38.1)	0.29
Age at onset (years) – number (% of column total)	0-0.25	19 (30.2)	6 (14.3)	13 (61.9)	
	0.25-1	2 (3.2)	2 (4.8)	0 (0)	
	1-5	22 (34.9)	19 (45.2)	3 (14.3)	
	6-12	14 (22.2)	9 (21.4)	5 (23.8)	
	13-18	6 (9.5)	6 (14.3)	0 (0)	
Family history positive / number with data available (%)		13 / 49 (26.5)	8 / 36 (22.2)	5 / 13 (38.5)	0.29
Consanguinity / number with data available (%)		11 / 50 (22.0)	5 / 36 (13.9)	6 / 14 (42.9)	0.052
Ethnicity (% of patients where data available)	**White**	38 (73.1)	28 (75.7)	10 (66.7)	
	Asian	10 (19.2)	7 (18.9)	3 (20.0)	
	Black African / Caribbean	3 (5.8)	1 (2.7)	2 (13.3)	0.61*
	Other	1 (1.9)	1 (2.7)	0 (0)	
	No ethnicity data available	11	5	6	
First biopsy findings (% of patients where data available)	**FSGS**	26 (59.1)	23 (69.7)	3 (27.3)	
	MCD	5 (11.4)	4 (12.1)	1 (9.1)	
	MHc	4 (9.1)	1 (3.0)	3 (27.3)	
	ESRF	2 (4.5)	0 (0)	2 (18.2)	
	FGGS	2 (4.5)	1 (3.0)	1 (9.1)	0.015†
	DMS	1 (2.3)	0 (0)	1 (9.1)	
	Collapsing glomerulopathy	1 (2.3)	1 (3.0)	0 (0)	
	Other	3 (6.8)	3 (9.1)	0 (0)	
	No biopsy data available / Not biopsied	19	9	10	
Pattern of steroid resistance (%)	**Presumed**	23 (36.5)	7 (16.7)	16 (76.2)	
	Primary	34 (54.0)	29 (69.1)	5 (23.8)	
	Secondary	4 (6.4)	4 (9.5)	0 (0)	
	Not tried	2 (3.2)	2 (4.8)	0 (0)	

Percentages are calculated for column totals. p values are for the comparison between genetic and non-genetic disease.

* For chi-squared analysis, "Black African / Caribbean" and "Other" were combined into one group

† For chi-squared analysis, "MHc", "ESRF", "FGGS", "DMS", "Collapsing glomerulopathy" and "Other" were combined into one group

Legend: DMS, diffuse mesangial sclerosis; FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease; MHc, mesangial hypercellularity; TBMN, thin basement membrane nephropathy

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For patients with available data, the group of 63 patients with CGT results had a significantly higher frequency of positive family history (26.5% vs 11.8%, p = 0.022, Fisher's exact test) and consanguinity (22.0% vs 7.2%, p = 0.0066, Fisher's exact test) compared with the 187 WES group. There was also a higher frequency of CNS (19/63 [30.2%] vs 15/187 [8.0%]) and presumed steroid resistance (36.5% vs 13.5%, p = 0.0002, chi-squared, 2df for the comparison of presumed, primary and secondary steroid resistance between the two cohorts). Despite these contrasts there was no statistically significant difference in rates of genetic disease (33.3% of 63 vs 27.8% of 187, p = 0.43, Fisher's exact test). The difference in clinical characteristics may partly be due to clinicians referring patients for CGT where there was a perceived higher prior probability of genetic disease. It is also likely that WES had a higher detection rate than single gene or gene panel testing.

For analysis of ethnicity, "Indian", "Pakistani", "Bangladeshi" and "Asian" were combined into one group, and "Mixed" and "Other" into a second group. There was no significant difference in ethnic groups between the 187 WES and 63 CGT cohorts (p = 0.48, chi-squared, 3df). For analysis of initial biopsy findings, four groups were used: FSGS, MCD, MHc and Other. There was no significant difference between the two cohorts (p = 0.23, chi-squared, 3df).

3.3.2 Medications administered and completeness of response data

The number of treatments received by patients in the WES group is shown in Table 3.3 and by patients in the CGT group in Table 3.4.

Table 3.3: Number of treatments and availability of outcome response data in WES group of patients

Groups	Subgroups	Number of patients	Number of treat-ments	Number of patients with outcome data	Number of treat-ments with outcome data
Total cohort		187	-	-	-
Not receiving ACEi/ARB or IIS (%)	**Total**	29 / 187 (15.5)	-	-	-
Reason for no ACEi/ARB or IIS	**CNS**	9	-	-	-
	CKD/ESRF at presentation	8	-	-	-
	Syndromic	3	-	-	-
	Steroid-sensitive	1	-	-	-
	Familial	1	-	-	-
	No medication data	7	-	-	-
Total receiving treatments (%)	**ACEi/ARB or IIS**	158 / 187 (84.5)	407	152 / 158 (96.2)	371 / 407 (91.2)
Grouped by patients (%)	**ACEi/ARB only**	19 / 158 (12.0)	-	-	-
	IIS only	64 /158 (40.5)	-	-	-
	ACEi/ARB and IIS	75 / 158 (47.5)	-	-	-
Grouped by treatments (%)	**All ACEi/ARB**	94 / 158 (59.5)	113 / 407 (27.8)	79 / 94 (84.0)	95 / 113 (84.1)
	All IIS	139 / 158 (88.0)	294 / 407 (72.2)	135 / 139 (97.1)	276 / 294 (93.9)

Table 3.4: Number of treatments and availability of outcome response data in CGT group of patients

Groups	Subgroups	Number of patients	Number of treat-ments	Number of patients with outcome data	Number of treat-ments with outcome data
Total cohort		63	-	-	-
Not receiving ACEi/ARB or IIS (% of 63 patients)	**Total**	19 / 63 (30.2)	-	-	-
Reason for no ACEi/ARB or IIS	**CNS**	13	-	-	-
	CKD/ESRF at presentation	3	-	-	-
	No medication data	3	-	-	-
Total receiving treatments (% of 63 patients or 95 treatments)	**ACEi/ARB or IIS**	44 / 63 (69.8)	95	38 / 44 (86.4)	81 / 95 (85.3)
Grouped by patients (%)	**ACEi/ARB only**	12 / 44 (27.3)	-	-	-
	IIS only	14 / 44 (31.8)	-	-	-
	ACEi/ARB and IIS	18 / 44 (40.9)	-	-	-
Grouped by treatments (%)	**All ACEi/ARB**	30 / 44 (68.2)	35 / 95 (36.8)	25 / 30 (83.3)	28 / 35 (80.0)
	All IIS	32 / 44 (72.7)	60 / 95 (63.2)	31 / 32 (96.9)	53 / 60 (88.3)

The proportion of patients not receiving ACEi/ARB or IIS in the CGT group was higher than in the WES group (30.2% vs 15.5%) broadly as a result of the higher number of patients with CNS in the former cohort. In both groups, of all patients receiving any medication the majority were given at least one IIS drug

(88.0% and 72.7%). On average, each patient received 2 different IIS treatments. Almost half (47.5% and 40.9%) received both ACEi/ARB and IIS at some stage.

For all treatments that were administered, the completeness of response outcomes was above 85% in both cohorts. The 371 treatments with known outcomes were given to 152 patients in the WES group and the 81 treatments were given to 38 patients in the CGT group. The clinical profiles of these patients are summarised in Figure 3.2.

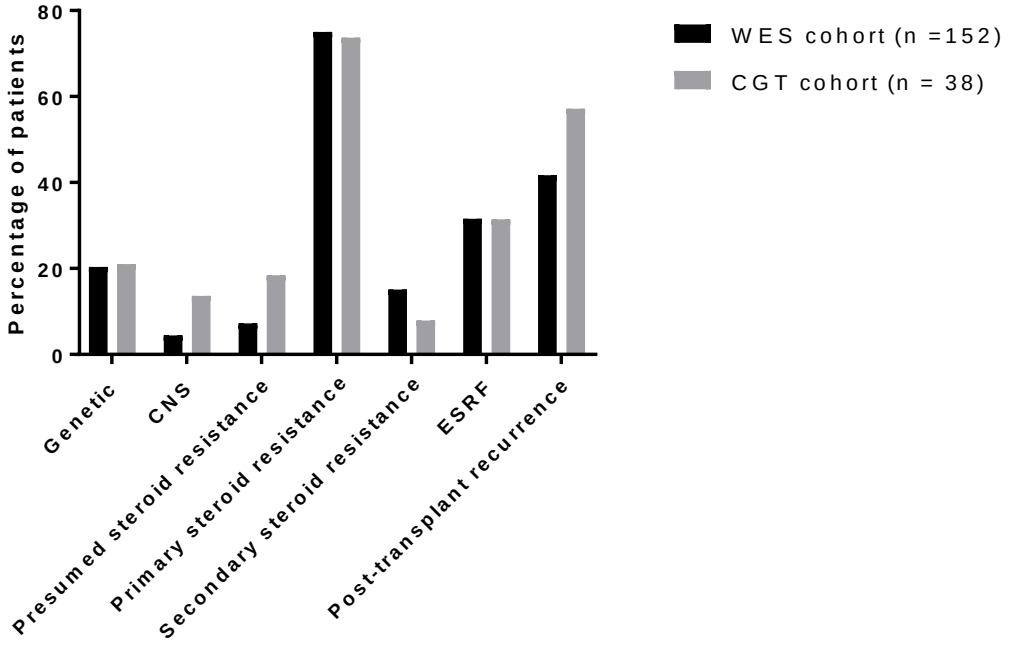

Figure 3.2: Clinical characteristics of patients for whom medication and response data were known

ESRF/transplant status were unknown for 3 patients in the CGT cohort. All other data in both cohorts were complete.

In general, the characteristics of patients in the two cohorts were similar, although there was a higher frequency of CNS and presumed steroid resistance in the CGT cohort. The apparent higher proportion of post-transplant recurrence in

this group (57.1%) in fact represented 4 of 7 patients and was not significantly different from 41.7% in the WES group (p = 0.68, Fisher's exact test).

In the subsequent assessment of responses to individual medications, data from all 190 patients from both cohorts were pooled and analysed together.

3.3.3 Response to immunosuppressive medications in the whole cohort

Of the 190 patients, 166 were given a total of 329 IIS treatments for which outcome data were available. The numbers of drug treatments and response rates are shown in Figure 3.3.

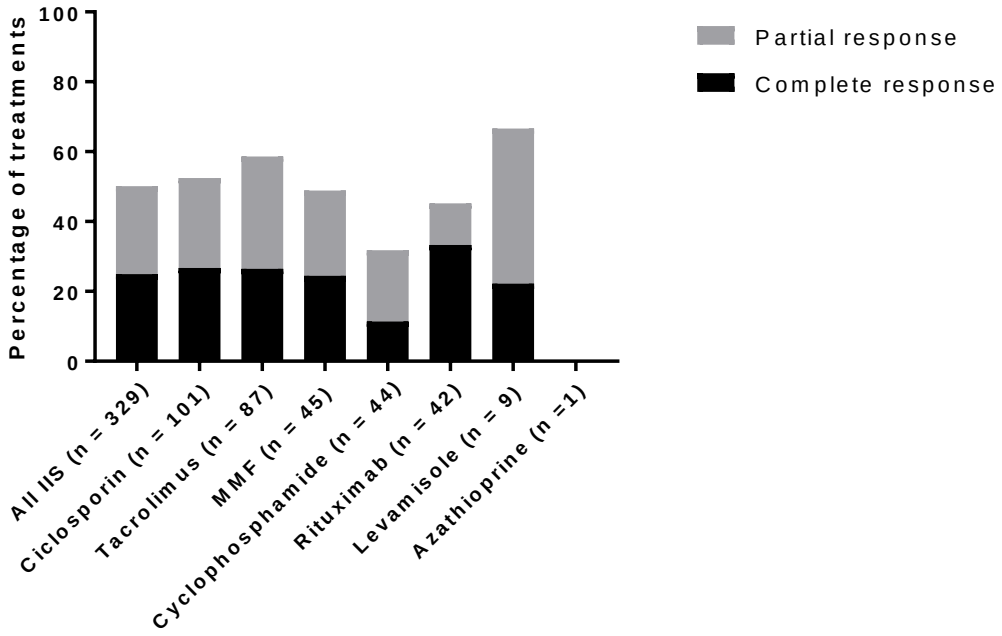

Figure 3.3: Response to intensified immunosuppressive (IIS) medications
The number of treatments administered is given in brackets

Around 50% of patients achieved either CR or PR when treated with each of ciclosporin, tacrolimus, MMF or rituximab. However, the proportion of patients achieving either CR or PR was lower at 31.8% for cyclophosphamide and only 11.4% achieved CR.

3.3.4 Response to first immunosuppressive medications in the whole cohort

Although the average number of IIS treatments for which outcome data were known was 2.0 per patient, the actual number of treatments administered varied between 1 and 5 and the distribution is shown in Table 3.5.

Table 3.5: Number of immunosuppressive treatments administered per patient

Number of IIS treatments administered per patient	Number of patients
1	72
2	50
3	25
4	13
5	6
Total	166

Since the second or subsequent IIS treatments may have been used in patients who had already failed the first therapy, the analysis of response rate may be confounded by the sequence in which clinicians chose to administer the medications. In order to avoid this potential bias, response rates to only the first treatment were examined.

Ciclosporin was the first treatment in 76 patients (45.8%), tacrolimus in 39 (23.5%) and cyclophosphamide in 33 (19.9%) with the other medications being used in fewer than 10 patients each. While MMF and rituximab were relatively frequently used in the entire cohort, they represented only a relatively small proportion of first-line IIS therapy. The responses to all initial treatments are shown in Figure 3.4.

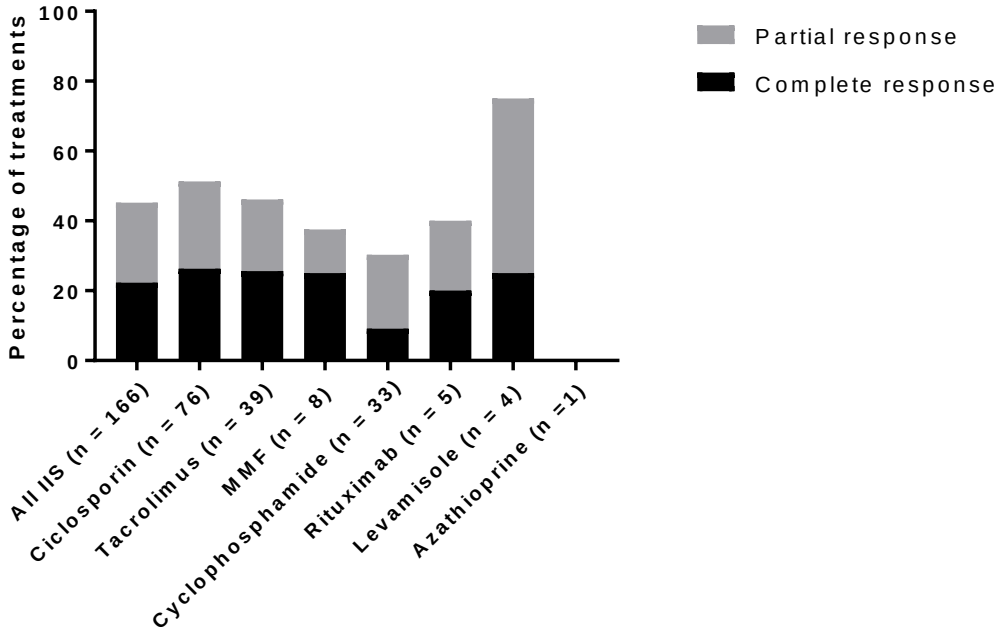

Figure 3.4: Response to first-administered intensified immunosuppressive (IIS) medications

Of the three most frequently-used first-line treatments, ciclosporin and tacrolimus appeared to have similar levels of response (CR in 26.3% and 25.6% of patients respectively). Both had a higher response than cyclophosphamide (9.1% of patients achieved CR), although not statistically significant in the case of

tacrolimus ($p = 0.045$ for cyclophosphamide versus ciclosporin; $p = 0.12$ for cyclophosphamide versus tacrolimus).

Other patient characteristics and long-term outcomes grouped by response to first IIS treatment are shown in Table 3.6.

Table 3.6: Characteristics and long-term outcomes of patients stratified by response to first immunosuppressive treatment

		Total with outcomes	Complete	Partial	No	N/A
Number of patients		166	37	38	91	5
First-line IIS treatment	Ciclosporin	76	20	19	37	3
	Tacrolimus	39	10	8	21	0
	MMF	8	2	1	5	0
	Cyclo-phosphamide	33	3	7	23	2
	Rituximab	5	1	1	3	0
	Levamisole	4	1	2	1	0
	Azathioprine	1	0	0	1	0
Age at onset (years) – number (% of column total)	0-0.25	2 (1.2)	0 (0)	1 (2.6)	1 (1.1)	0 (0)
	0.25-1	3 (1.8)	0 (0)	2 (5.3)	1 (1.1)	0 (0)
	1-5	96 (57.8)	26 (70.3)	24 (63.2)	46 (50.6)	4 (80.0)
	6-12	51 (30.7)	11 (29.7)	7 (18.4)	33 (36.3)	0 (0)
	13-18	14 (8.4)	0 (0)	4 (10.5)	10 (11.0)	1 (20.0)
Pattern of steroid resistance	Presumed SR	4 (2.4)	0 (0)	1 (2.6)	3 (3.3)	0 (0)
	Primary SR	133 (80.1)	26 (70.3)	30 (79.0)	77 (84.6)	2 (40.0)
	Secondary SR	26 (15.7)	10 (27.0)	6 (15.8)	10 (11.0)	3 (60.0)
	SSNS	3 (1.8)	1 (2.7)	1 (2.6)	1 (1.1)	0 (0)
Number (%) with genetic disease		24 (14.5)	1 (2.7)	9 (23.7)	14 (15.4)	1 (20.0)
Number (%) who developed ESRF		50 / 164 (30.5)	2 / 36 (5.6)	4 / 38 (10.5)	44 / 90 (48.9)	3 / 5 (60.0)
Number (%) transplanted		35 / 164 (21.3)	1 / 36 (2.8)	3 / 38 (7.9)	31 / 90 (34.4)	3 / 5 (60.0)
Number (% of those transplanted) with post-transplant recurrence		19 / 35 (54.3)	1 / 1 (100)	0 / 3 (0)	18 / 31 (58.1)	2 / 3 (66.6)

Legend: ESRF, end stage renal failure; IIS, intensified immunosuppressive; MMF, mycophenolate mofetil; N/A, outcome data not available; SR, steroid resistance

As might be expected, only a small number of patients with CNS or infantile-onset were given IIS treatment. More detailed analysis of responses for patients stratified by genetic disease will be discussed in Section 3.3.5, stratified by pattern of steroid resistance in Section 3.3.6, stratified by first biopsy findings in Section 3.3.7 and the long-term outcomes in Section 3.3.9.

3.3.5 Response to immunosuppressive medications stratified by genetic disease

Of 166 patients who were treated with IIS medications and with outcome data available, 24 (14.5%) had genetic disease. When only *first-line* ISS treatments were considered (Table 3.6), CR was seen in 4.2% of patients and PR in 37.5% of patients. These were significantly different from the corresponding responses for patients with non-genetic disease (25.4% achieved CR and 20.4% achieved PR, $p = 0.033$, chi-squared, 2df). However, the proportion of patients achieving either CR or PR was not significantly different between patients with genetic versus non-genetic disease (41.7% versus 45.8% respectively, $p = 0.83$, Fisher's exact test).

In total, 40 IIS medications were administered to the 24 patients with genetic disease (1.7 medications/patient) and 289 were given to 142 patients with non-genetic disease (2.0 medications/patient). The numbers of drug treatments and response rates are shown in Figure 3.5.

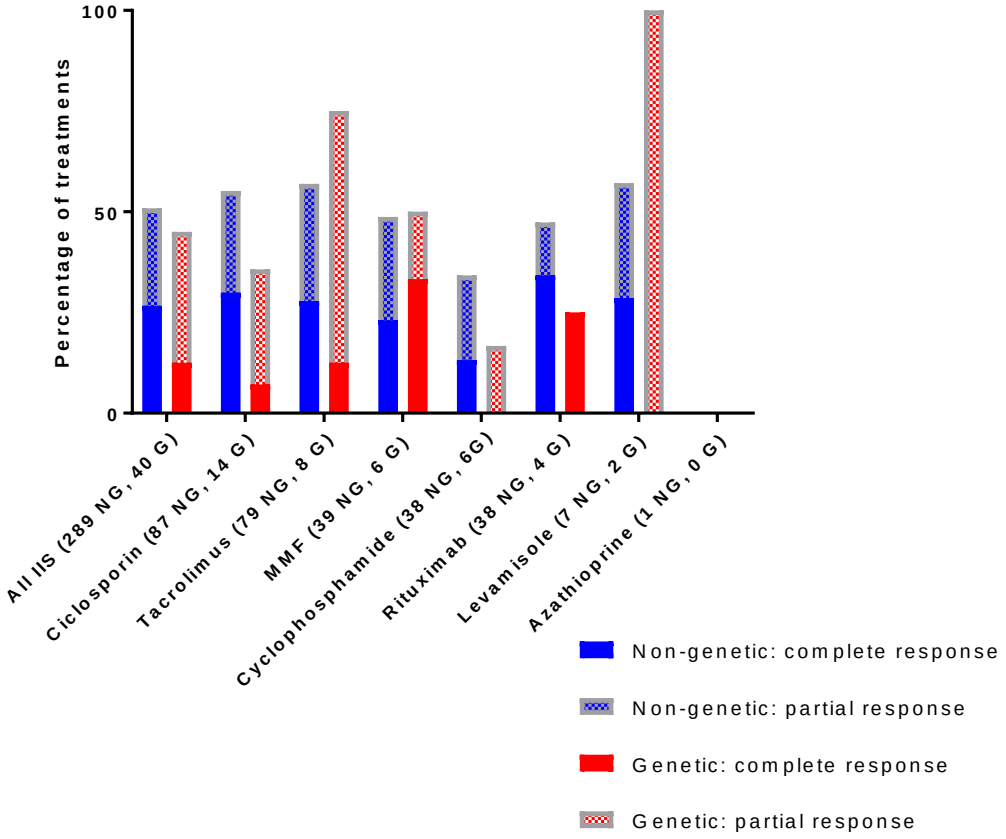

Figure 3.5: Response to intensified immunosuppressive (IIS) medications in patients with genetic or non-genetic disease

Details in brackets are the total number of treatments given to patients with genetic (G) or non-genetic (NG) disease

When considering *all* IIS treatments, 26.6% of patients with non-genetic disease had CR and 24.2% of patients had PR. 12.5% of patients with genetic disease had CR and 32.5% of patients had PR. There was no significant difference in the number of patients achieving complete or partial remission between the two groups ($p = 0.50$, Fisher's exact test); nor was there a difference in the number of patients achieving complete remission alone ($p = 0.053$).

When considering each drug separately, there was no significant difference between patients with genetic disease and those with non-genetic disease in relation to the number achieving CR or PR when treated with

ciclosporin (35.7% vs 55.2%, p = 0.25), tacrolimus (75.0% vs 57.0, p = 0.46), MMF (50.0% vs 48.7%, p > 0.99), cyclophosphamide (16.7% vs 34.2%, p = 0.65) or rituximab (25.0% vs 47.4%, p = 0.61). In general, the number of patients with genetic disease receiving IIS medications was small and the numbers given levamisole and azathioprine were too small for analysis.

Two patients with genetic disease appeared to achieve CR with IIS medications. The details of these patients are shown in Table 3.7 with full clinical information available in Table 10.1 and Table 10.2 in the Appendices.

Table 3.7: Patients with genetic disease who had complete response to immunosuppression

Patient	Gene	Gender	Age at onset (years)	Resistance to steroids	1st biopsy	CKD stage	Extra-renal phenotype	Length of follow up (years)	Medications for which there was a complete response
353	*NPHS1*	M	0.1	sensitive (frequently relapsing)	Other	1	No	17.7	Ciclosporin MMF Rituximab
7656	*WT1*	M	3.2	primary	DMS	2	Denys-Drash syndrome	1.6	MMF Tacrolimus

According to RaDaR data, patient 7656 was not on ACEi or ARB but was started on MMF and tacrolimus concomitantly which was confirmed by the local research team. Patient 353 was treated initially with ciclosporin, started MMF over 3 years later and was given rituximab 4 years after that. Unusually this patient had a congenital onset of disease but was steroid-sensitive and was found to have compound heterozygous possibly-pathogenic variants in *NPHS1* (c.1747G>A:p.Glu583Lys; c.2734G>A:p.Ala912Thr). The c.1747G>A variant had a frequency in EXaC of 0.0001065 and the other variant was not reported in that database. Neither had been reported previously in patients and in the absence

of parental samples from patient 353, it was not possible to confirm that the variants were in *trans* (personal communication, Dr Agnieszka Bierzynska).

Nine patients with genetic disease appeared to show PR to the first-administered IIS. These patients are detailed in Table 3.8 with complete clinical and outcome information in the tables at the Appendices. None has progressed to ESRF, although follow-up for three patients was under 2 years.

Table 3.8: Patients with genetic disease who had partial response to first immunosuppression

Patient	Gene	Gender	Age at onset (years)	Resistance to steroids	1st biopsy	CKD stage	Extra-renal phenotype	Length of follow up (years)	Medications for which there was partial response
353	*NPHS1*	M	0.1	sensitive (frequently relapsing)	Other	1	No	17.7	Levamisole
495	*NPHS1*	F	2.0	primary	MCD	2	No	17.8	MMF
514	*SMARCAL1*	M	7.1	primary	FSGS	1	No	0.4	Ciclosporin
687	*CRB2*	F	0.8	presumed	MCD	1	No	4.0	Levamisole
729	*NPHS2*	M	7.9	primary	Other	2	Asthma	2.8	Tacrolimus
731	*MAGI2*	M	0.3	primary	MCD	1	Pyloric stenosis, Poly-dactyly, Thrombo-cytosis	11.7	Ciclosporin
770	*COL4A3*	F	7.9	primary	FSGS	1	No	2.5	Ciclosporin
811	*WT1*	F	3.8	primary	Alports	1	Chronic cough and diarrhoea, Frasier syndrome	1.1	Tacrolimus
900	*LMX1B*	F	14.9	primary	FSGS	1	Delayed puberty	1.1	Tacrolimus

In 2 patients (495 and 900) the response was based on the opinion of the treating clinician. In the remaining 7, plasma albumin was ≥ 30g/L and for the 6 with proteinuria data, uPCR or dipstick proteinuria decreased from > 200

mg/mmol to < 80 mg/mmol or 2+ within the period following the start of IIS therapy. Based on RaDaR data, one patient (514) started enalapril shortly after ciclosporin and the observed partial response may, therefore, have been due to the combination of treatment rather than IIS alone.

Three of the patients (353, 687, 731) had congenital or infantile-onset disease and may not have been expected to respond to IIS drugs. Two of these were subsequently found to have likely-pathogenic variants in genes that were novel or rarely associated with childhood SRNS (*MAGI2* in 731 and *CRB2* in 687). The other patients' disease was caused by a range of different genes and showed response to various immunosuppressive drugs and it is, therefore, difficult to draw any broader conclusions.

3.3.6 Response to immunosuppressive medications stratified by pattern of steroid resistance

Of the 166 patients who were given IIS medications and with treatment responses available for analysis, 4 had presumed, 133 had primary and 26 had secondary steroid resistance. The remaining 3 had SSNS and were not included in the subsequent analysis.

As discussed in Chapter 2, patients with non-genetic disease and presumed/primary steroid resistance (NGPPSR, 114 patients) may represent a subgroup distinct from those with secondary steroid resistance (SSR, 26 patients), therefore the responses of these groups to IIS were compared. When considering only *first-line* IIS medications (Table 3.6), CR was achieved in 25 patients with NGPPSR (21.9%), and PR in 23 patients with NGPPSR (20.2%) compared with

10 patients with SSR who achieved CR (38.5%) and 6 patients with SSR who achieved PR (23.1%) (p = 0.14, chi-squared, 2df). There was no significant difference in the number of patients who achieved CR or PR between the two groups (p = 0.08, Fisher's exact test).

In total, 140 patients received 285 IIS medications. The numbers and response rates to different treatments is shown in Figure 3.6.

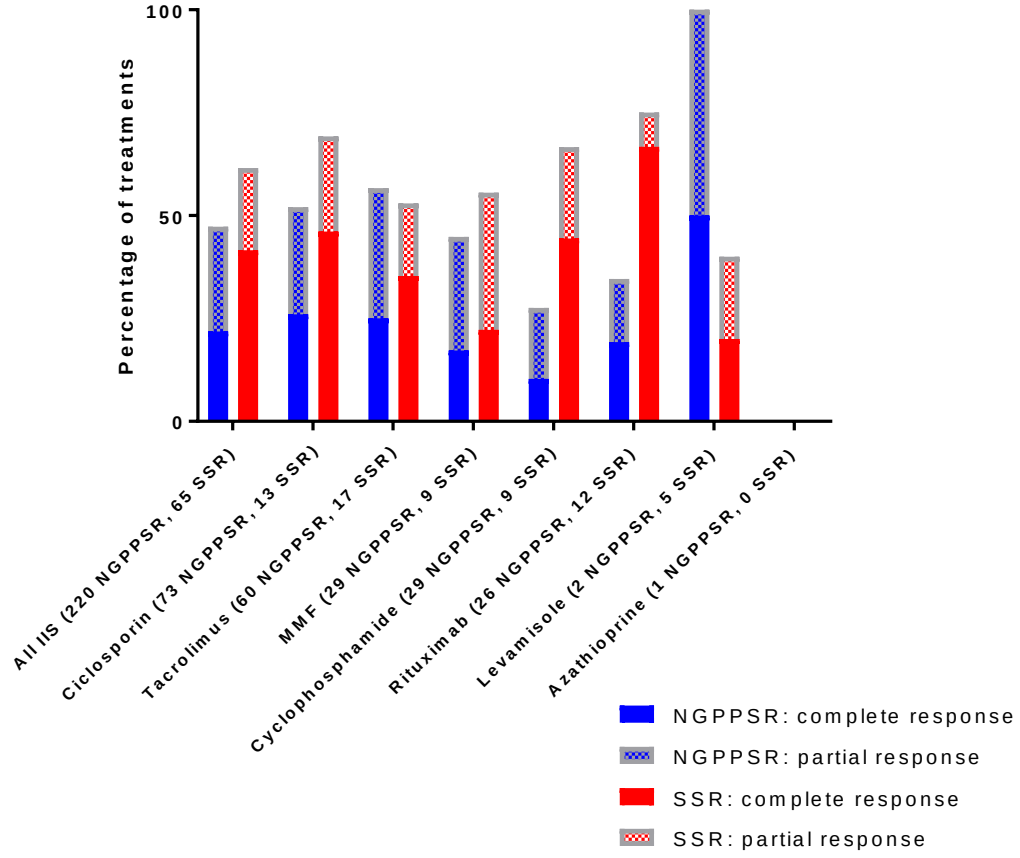

Figure 3.6: Response to intensified immunosuppressive (IIS) medications in patients with non-genetic presumed/primary steroid resistance and secondary steroid resistance

Details in brackets are the total number of treatments given to patients with non-genetic presumed/primary steroid resistance (NGPPSR) or secondary steroid resistance (SSR)

Overall, when considering *all* 285 IIS treatment episodes, the frequency of CR was 21.8% and PR was 25.5% for patients with NGPPSR compared with 41.5% and 20.0% respectively for patients with SSR. In patients with SSR, the number of treatment episodes leading to CR was significantly higher than in patients with NGPPSR ($p = 0.0022$, Fisher's exact test). Likewise, the number of treatment episodes leading to CR or PR was higher in patients with SSR compared with those with NGPPSR ($p = 0.049$). Analysis of individual medications showed that rituximab led to CR in 66.7% of patients with SSR compared with 19.2% of patients with NGPPSR ($p = 0.0086$). Likewise, rituximab led to CR or PR in 75.0% of patients with SSR compared with 34.6% of patients with NGPPSR ($p = 0.035$). There was no significant difference in responses between the groups to ciclosporin, tacrolimus, MMF or cyclophosphamide.

3.3.7 Response to immunosuppressive medications stratified by first biopsy findings

Of 166 patients who were given IIS medication and with treatment responses available, 162 had biopsies performed with results for analysis. FSGS was seen in 96 (59.3%) first biopsies, MCD in 41 (25.3%), MHc in 11 (6.8%) and other findings in 14 (8.6%). The treatments given and clinical characteristics of the groups are shown in Table 3.9.

Table 3.9: First immunosuppressive treatments, characteristics and long-term outcomes of patients stratified by first biopsy findings

		Total biopsied with outcomes	FSGS	MCD	MHc	Other
Number of patients		164	96	41	11	14
First-line IIS treatment	Ciclosporin	75	47	15	7	6
	Tacrolimus	37	22	9	1	5
	MMF	7	2	4	0	1
	Cyclo-phosphamide	33	20	10	2	1
	Rituximab	5	3	1	1	0
	Levamisole	4	1	2	0	1
	Azathioprine	1	1	0	0	0
Age at onset (years) – number (% of column total)	0-0.25	2 (1.2)	0 (0)	0 (0)	1 (9.1)	1 (7.1)
	0.25-1	3 (1.8)	0 (0)	2 (4.9)	0 (0)	1 (7.1)
	1-5	93 (57.4)	52 (54.2)	24 (58.5)	8 (72.7)	9 (64.3)
	6-12	50 (30.9)	33 (34.4)	13 (31.7)	1 (9.1)	3 (21.4)
	13-18	14 (8.6)	11 (11.5)	2 (4.9)	1 (9.1)	0 (0)
Pattern of steroid resistance	Presumed SR	4 (2.5)	1 (1.0)	1 (2.4)	2 (18.2)	0 (0)
	Primary SR	131 (80.9)	83 (86.5)	29 (70.7)	8 (72.7)	11 (78.6)
	Secondary SR	24 (14.8)	11 (11.5)	10 (24.4)	1 (9.1)	2 (14.3)
	SSNS	3 (1.8)	1 (1.0)	1 (2.4)	0 (0)	1 (7.1)
Number (%) with genetic disease		24 (14.8)	12 (12.5)	5 (12.2)	3 (27.3)	4 (28.6)
Number (%) who developed ESRF		50 / 161 (31.1)	34 / 95 (35.8)	11 / 41 (26.8)	3 / 11 (27.3)	2 / 14 (14.3)
Number (%) transplanted		35 / 161 (21.7)	26 / 95 (27.4)	7 / 41 (17.1)	1 / 11 (9.1)	1 / 14 (7.1)
Number (% of those transplanted) with post-transplant recurrence		19 / 35 (54.3)	11 / 26 (42.3)	6 / 7 (85.7)	1 / 1 (100)	1 / 1 (100)

.

Considering only the groups with FSGS and MCD, there was no significant difference between the specific first IIS medication administered (p = 0.26, chi-squared, 4df, with rituximab, levamisole and azathioprine combined into one group); no difference in age at onset (p = 0.41 chi-squared, 2 df with 0-5 years as one group); no difference in presumed/primary versus secondary steroid resistance (p = 0.068, Fisher's exact test); no difference in frequency of genetic disease (p > 0.99, Fisher's exact test) and no difference in the proportion reaching ESRF (p = 0.33, Fisher's exact test). There was also no difference in the proportion who were transplanted (p = 0.28, Fisher's exact test) or in post-transplant recurrence (p = 0.085, Fisher's exact test).

The responses to *first-line* IIS treatment based on first biopsy findings are shown in Figure 3.7.

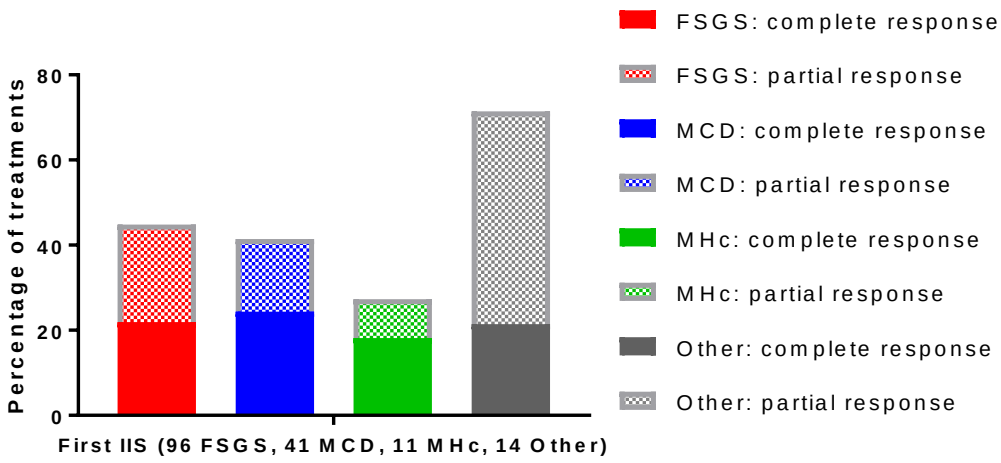

Figure 3.7: Response to first intensified immunosuppressive (IIS) medications in patients stratified by first biopsy findings

Details in brackets are the total number of patients with each biopsy finding

Overall, there was no significant difference in response to *first-line* IIS between the groups based on first biopsy findings ($p = 0.19$, chi-squared, 6df). Of the group with "other" biopsy findings 21.4% of patients achieved CR and 50% of patients achieved PR. These proportions were significantly different when compared with the responses of the other three groups (FSGS + MCD + MHc) combined ($p = 0.032$, chi-squared, 2df). "Other" findings included four with mesangial proliferation, two with focal global glomerulosclerosis, two with C1q nephropathy, one each with DMS, collapsing glomerulopathy, Alport's, thin membrane disease, IgA nephropathy, and one unspecified.

When considering only the two most prevalent histology findings (FSGS and MCD), there were no significant differences in patients achieving CR (21.9% in patients with FSGS versus 24.4% in those with MCD, $p = 0.82$, Fisher's exact test) or in those who achieved either CR or PR (44.8% in patients with FSGS versus 41.5% in patients with MCD, $p = 0.85$, Fisher's exact test).

In total, the 162 patients with reported biopsy results were given 320 ISS medications. The numbers and responses to *all* IIS treatments stratified by biopsy findings are shown in Figure 3.8.

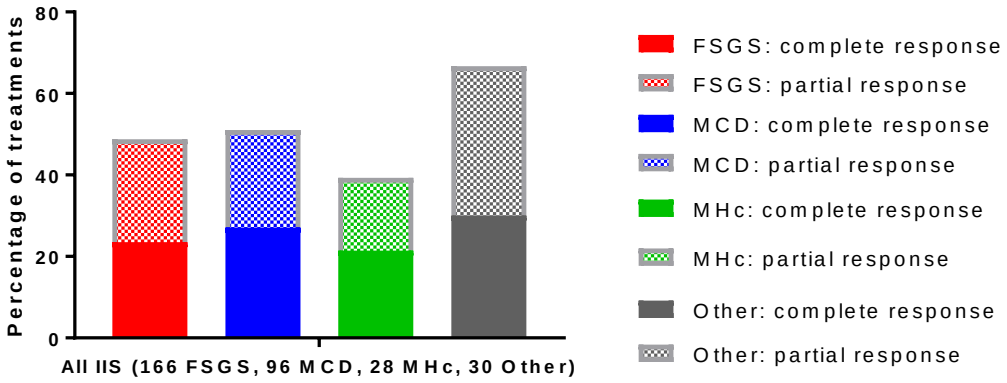

Figure 3.8: Response to all intensified immunosuppressive (IIS) medications in patients stratified by first biopsy findings

Details in brackets are the total number of treatments received by patients with each of the biopsy findings

When considering the response in patients to *all* IIS treatments, there was no significant difference between groups with different biopsy findings (p = 0.50, chi-squared, 6df). In this analysis, patients with "other" findings on biopsy had no difference in response compared with the other groups (p = 0.15, chi-squared, 2df). There were no differences in either the numbers achieving CR or the numbers achieving either CR or PR between patients with FSGS and those with MCD (p = 0.55 and p = 0.80 respectively, Fisher's exact test).

3.3.8 Prior response to immunosuppressive medications in patients with post-transplant disease recurrence

Although post-transplant disease recurrence cannot be used as a predictive marker for direct management in clinical practice, as discussed previously, it can give an insight into disease biology and indicate patients likely to have a circulating factor pathogenesis. It has been speculated that the circulating factor(s) is immune-derived.

Of the 166 patients in the current analysis, 35 received a transplant and recurrence occurred in 19 (54.3%). These included 17 with primary steroid resistance (none genetic) and 2 with secondary steroid resistance. This group of 19 patients received 43 IIS treatments. The 16 who did not suffer post-transplant recurrence included 14 with primary steroid resistance (7 genetic) and 2 with secondary steroid resistance. Excluding patients with genetic disease, the 9 patients received 13 IIS treatments.

The numbers of patients receiving individual medications were too small for statistical analysis. Of the 43 treatment episodes relating to 19 patients who were later treated for post-transplant disease recurrence, CR was achieved in 4.7% of treatment episodes and PR in 4.7% of treatment episodes. Among the 13 treatments given to 16 patients with no post-transplant recurrence, the CR response rate was 0% and the PR rate was 23.1% of treatment episodes. None of the latter patients who achieved PR concurrently started treatment with ACEi or ARB.

3.3.9 Long term outcomes in patients with SRNS stratified by response to first immunosuppressive treatment

The response to initial immunosuppression is sometimes used by clinicians when offering prognostic information to patients. The responses to first-line IIS treatments were discussed earlier in Section 3.3.4. The long-term outcomes including progression to ESRF, transplantation and post-transplant recurrence were detailed above in Table 3.6.

Among the 166 patients treated with IIS drugs and with known outcomes, the CKD status at most recent follow-up was known for 164. For these, the total

follow-up time was 844.4 patient-years with median 4.3 years (IQR 2.2-7.5, range 0.1-17.8 years).

The frequency of progression to ESRF clearly increased from 5.6% in those with CR, to 10.5% in those with PR and to 48.9% in those with no response to first IIS treatments ($p < 0.0001$, chi-squared, 2df). The frequency of transplantation was also significantly higher in patients failing to respond to the first treatment ($p < 0.0001$, chi-squared, 2df).

Patients with no response to first IIS therapy had significantly more rapid progression to ESRF as shown in the Kaplan-Meier survival curves with $p < 0.0001$ by log-rank (Mantel-Cox) test (Figure 3.9). The five-year ESRF-free survival rates were 93.8%, 90.3% and 60.0% for patients with CR, PR and no response respectively. The corresponding 10-year rates were 93.8%, 81.3% and 25.7%.

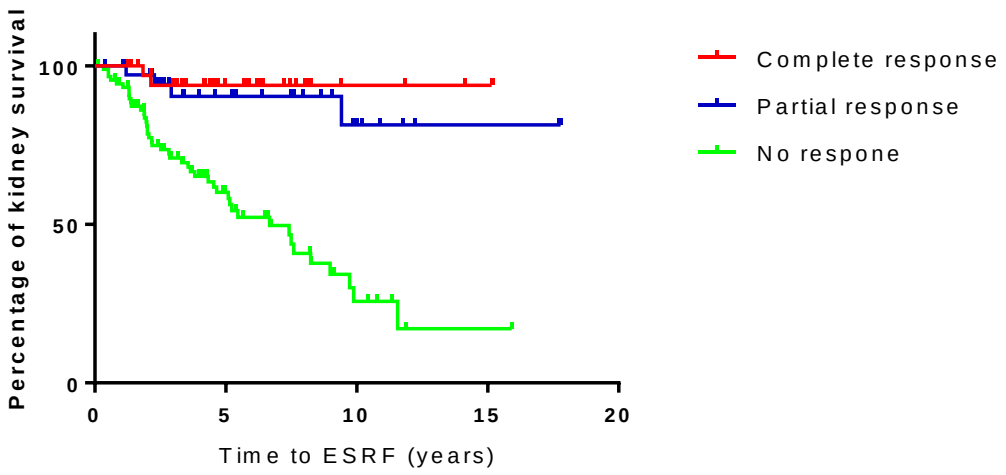

Figure 3.9: Kidney survival analysed by response to first immunosuppressive treatment

Numbers below the figure are the numbers of patients at risk at each time point

Post-transplantation disease recurrence occurred in 25% (1/4 patients) who achieved either CR or PR versus 58.1% (18/31 patients) who did not respond to IIS, although this difference was not statistically significant ($p = 0.31$, Fisher's exact test). As discussed in Chapter 2 (Section 2.3.6), genetic disease is a strong predictor of not developing disease recurrence post-transplant. In the subgroup without genetic disease, recurrence occurred in 25% (1/4 patients) who achieved either CR or PR versus 75% (18/24 patients) who did not respond to IIS ($p = 0.084$, Fisher's exact test).

3.3.10 Characteristics and outcomes in patients treated with rituximab

Of all IIS treatments, whether analysed collectively or individually, and however the patients were stratified, CR was generally seen in below 30% of patients. The most notable exception, as noted in Section 3.3.6, was rituximab used in patients with SSR where the frequency of CR was 66.7% (8 of 12 patients). By definition, patients with SSR were at one point steroid-sensitive and it may be that rituximab was being used to manage SDNS or FR-SSNS. In order to understand the implications of these findings, patients treated with rituximab were studied in more detail (Table 3.10).

Table 3.10: Characteristics and long-term outcomes of patients treated with rituximab stratified by response

		Total with outcomes	Complete	Partial	No	N/A
Total patients (%)		42	14 (33.3)	5 (11.9)	23 (54.8)	3
Age at onset (years) – number	0-0.25	1	1	0	0	0
	0.25-1	0	0	0	0	0
	1-5	25	10	3	12	2
	6-12	10	3	0	7	1
	13-18	6	0	2	4	0
Pattern of steroid resistance (%)	**Primary**	29 (69.1)	5 (35.7)	4 (80.0)	20 (87.0)	2 (66.7)
	Secondary	12 (28.6)	8 (57.1)	1 (20.0)	3 (13.0)	1 (33.3)
	SSNS	1 (2.4)	1 (7.1)	0 (0)	0 (0)	
First biopsy findings (% of patients where data available)	**FSGS**	14 (34.2)	3 (21.4)	3 (60.0)	8 (36.4)	0 (0)
	MCD	18 (43.9)	7 (50.0)	1 (20.0)	10 (45.5)	3 (100)
	MHc	6 (14.6)	2 (14.3)	1 (20.0)	3 (13.6)	0 (0)
	Other	3 (7.3)	2 (14.3)	0 (0)	1 (4.6)	0 (0)
	No biopsy data available / Not biopsied	1	0	0	1	0
Number (%) with genetic disease		4 (9.5)	1 (7.1)	0 (0)	3 (13.0)	0 (0)
Number (%) who developed ESRF		14 / 42 (31.1)	1 / 14 (7.1)	1 / 5 (20.0)	12 / 23 (52.2)	0 (0)
Number (%) transplanted		7 / 42 (16.7)	0 / 14 (0)	0 / 5 (0)	7 / 23 (30.4)	0 (0)
Number (% of those transplanted) with post-transplant recurrence		5 / 42 (11.9)	-	-	5 / 7 (71.4)	-

Legend: N/A, patients treated with rituximab but for whom outcome data were not available. The numbers in brackets are the percentages of the column totals (except for the top row which shows percentages of the grand total).

Of all patients treated with rituximab, CR was seen in 33.3% and PR in 11.9%. CR was observed most frequently in patients with SSR and those with MCD. One patient with genetic disease appeared to have CR to rituximab (patient 353), but the clinical presentation in this case was of FR-SSNS and they were discussed in more detail previously in Section 3.3.5. Complete or partial response to rituximab was associated with a significantly lower frequency of progression to ESRF (10.5% versus 52.2%, $p = 0.008$) and no patients had been transplanted at most recent follow-up. One patient with CR to rituximab (patient 615) progressed to ESRF. Further review of clinical information revealed that the patient remained steroid-sensitive at the time of rituximab treatment but became steroid-resistant 9 months later before progressing to ESRF over the subsequent 4 months.

The timing of starting rituximab after diagnosis of nephrotic syndrome is shown in Figure 3.10.

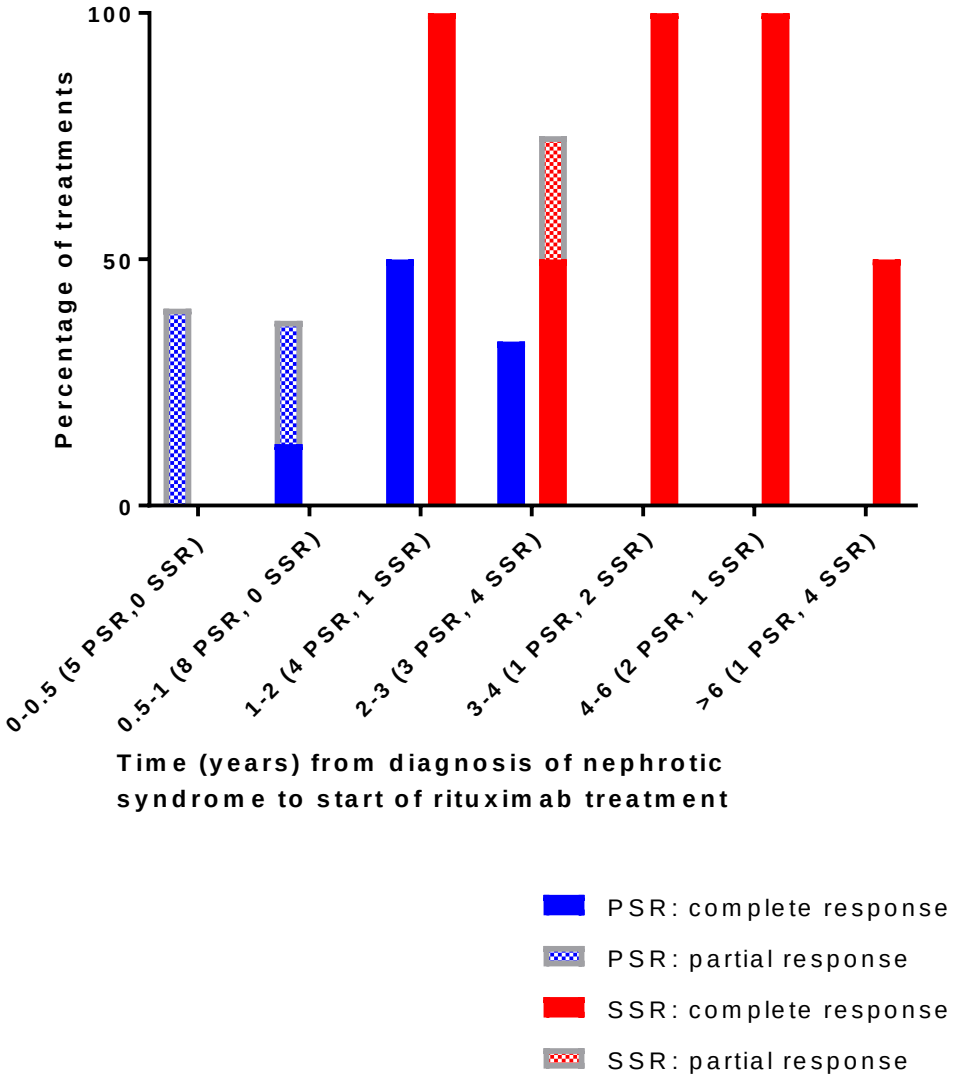

Figure 3.10: Response to rituximab in patients stratified by timing of treatment and pattern of steroid response

Legend: PSR, primary steroid resistance; SSR, secondary steroid resistance
The exact time between diagnosis and start of rituximab treatment was not known for 5 patients with PSR and so are not shown in this figure.

Thirteen patients with PSR were treated with rituximab within the first year of diagnosis but only 1 (7.7%) showed CR. None of the 4 patients with PSR with treatment onset after 3 years since diagnosis showed any response. Overall, patients with PSR who had complete or partial response had a younger median age at disease onset (2.6 versus 6.0 years, p = 0.39, Mann-Whitney test) and

shorter disease duration at time of treatment (0.6 versus 1.4 years, p = 0.19, Mann-Whitney test) but the differences were not significant.

All patients with SSR started rituximab more than 1 year after diagnosis. Nine (75%) had CR or PR. Among patients with SSR, there was no significant difference between patients achieving either CR or PR compared with those who did not respond to IIS in terms of median age of disease onset (4.0 versus 4.7 years, p = 0.86, Mann-Whitney test) or median time between diagnosis and treatment (3.3 versus 6.4 years, p = 0.28, Mann-Whitney test).

The responses of patients with SSR to rituximab were examined in greater detail. Of 12 patients treated, 8 had CR and one had PR. Using data available in RaDaR, among the former group of 8 patients it appeared that 3 (patients 413, 567, 615) had FR-SSNS or SDNS at the time they received rituximab treatment. The other 5 patients (457, 469, 475, 505, 552) had been classed as steroid-resistant (with failure to achieve complete remission of proteinuria on > 4 weeks of steroid treatment) prior to receiving rituximab but documented information suggested that there was still some responsiveness to steroids. The patient with PR (patient 427) was initially steroid-sensitive but became resistant by 3 months after diagnosis and was treated with rituximab 2.5 years later. The three patients with SSR who had no response to rituximab were treated 4 months, 1 year and 10 years respectively after becoming steroid-resistant and had received 2, 5 and 2 IIS drugs previously since original diagnosis with NS.

3.3.11 Response to ACEi and ARB in the whole cohort

In the whole cohort of 250 patients, based on data available in RaDaR, 124 patients received ACEi and/or ARB. A total of 148 drugs in these classes were

administered. As highlighted in the Methods, only the earliest-prescribed drug for each patient in each of the two classes were considered for analysis. Response data were available for 123 (83.1%) of these treatments which were given to 104 patients. Nineteen patients received both ACEi and ARB, 11 patients had ARB only and 74 had ACEi only. The number of treatments and responses are detailed in Table 3.11 and shown in Figure 3.11.

Table 3.11: Number of ACEi / ARB treatments administered and responses

		Total with outcomes	Complete	Partial	No	N/A
Number of treatments		123	19	29	75	25
First ACEi	**All ACEi**	93	15	22	56	17
	Captopril	8	1	0	7	0
	Enalapril	42	9	12	21	12
	Lisinopril	13	2	3	8	1
	Ramipril	4	0	0	4	2
	Not specified	26	3	7	16	2
First ARB	**All ARB**	30	4	7	19	8
	Irebesartan	1	0	0	1	1
	Losartan	22	4	5	13	6
	Valsartan	1	0	1	0	0
	Not specified	6	0	1	5	1

Legend: N/A, patients who received ACEi / ARB but for whom outcome data were not available

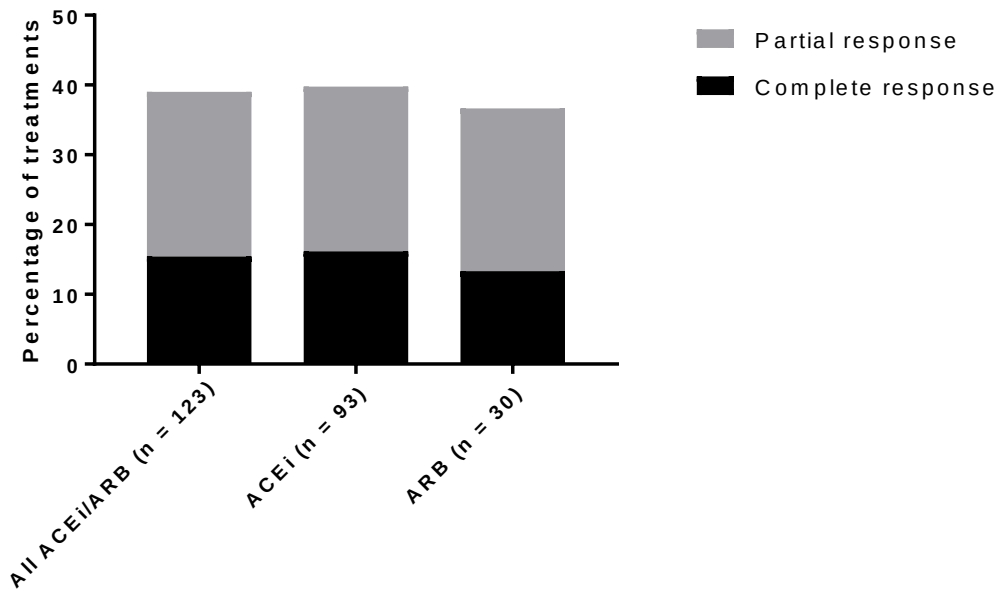

Figure 3.11: Response to first-administered ACEi and ARB
Figures in brackets are the total number of treatments

For all ACEi as a class, there were no significant differences in CR between patients with genetic versus non-genetic disease, with NGPPSR versus SSR, or with FSGS versus MCD. Considering ARB, there were no differences in CR between patients with genetic versus non-genetic disease, or with FSGS versus MCD. No patients with SSR received ARB.

3.4 Discussion

3.4.1 Findings of this study

The data presented here, from a UK cohort of 166 patients with SRNS/FSGS treated with IIS and with genetic testing results available, revealed that the most-frequent first-line medications were ciclosporin, tacrolimus and

cyclophosphamide with MMF and rituximab also given frequently as second-line or subsequent agents. Overall, the efficacy of the treatments was limited, with some response (either CR or PR) in approximately 50% of patients treated with ciclosporin, tacrolimus, MMF or rituximab. The response was lower at 31.8% for patients treated with cyclophosphamide.

An aim of this study was to ascertain whether stratification of patients by baseline characteristics could identify subgroups who were more responsive to IIS treatment. This chapter has examined genetics, pattern of steroid resistance and first biopsy findings in particular.

3.4.1.1 Genetic disease

In patients with genetic disease, confirmed by WES or CGT, there was a statistically significantly lower probability of achieving CR to first IIS compared with those without genetic disease (4.2% versus 25.4%). The differences were not significant, however, when comparing patients with or without genetic disease experiencing any response (either CR or PR) to IIS or when comparing responses to all IIS treatment episodes (as opposed to the first-administered). The PR rates in patients with genetic disease were comparatively high (37.5% for *first-line* IIS treatments and 32.5% for *all* treatment episodes). The definition of PR was ongoing proteinuria (uPCR > 20 mg/mmol or dipstick ≥ 1+) but plasma albumin > 25 g/L within 6 months of starting therapy. If plasma albumin was already > 25 g/L prior to starting treatment but remained above this in the following 6 months and proteinuria did not reach the threshold for CR, this was also classed as PR. Defined in this way, PR may have been relatively easy to achieve. However, as

the same criteria were applied to all patients, this would not explain differences between genetic and non-genetic patients.

As a retrospective cohort study, the decisions about which treatments to use, and when, were made by the treating clinicians. The majority of treatments examined in this analysis were started prior to 2014, whereas results for WES and CGT were becoming available after this time. Therefore, it is likely that most therapy decisions were being made without definite knowledge of genetic status. Of 166 patients included who received IIS, 24 (14.5%) had an identified genetic aetiology compared with 73 (29.2%) of the original cohort of 250 who had had genetic testing (see Figure 3.1). The response to IIS in patients with genetic disease in this study is, therefore, not representative of all inherited forms of SRNS. It is likely that clinicians selected patients to receive IIS based on clinical features which they associated with a higher chance of response to that treatment. For example, clinicians did not give IIS or ACEi/ARB to 22 patients with CNS (see Table 3.3 and Table 3.4) of whom 17 (77.3%) were confirmed to have genetic disease.

Of the cohort of 166 patients treated with IIS, 10 with genetic disease showed CR or PR. These patients had likely-pathogenic variants in 8 different genes and variously showed response to ciclosporin, tacrolimus, MMF, rituximab and levamisole. With such small patient numbers and diverse treatments, it is not possible to determine whether any specific treatment may be more effective in patients with genetic disease.

3.4.1.2 Pattern of steroid resistance

Patients with genetic disease were excluded from the analysis of response to IIS stratified by pattern of steroid resistance. This study has shown that patients with SSR treated with IIS had a significantly higher rate of response compared with those with NGPPSR. When responses to individual IIS medications were analysed, this significant difference held for rituximab but not others.

Further examination of patients with SSR given rituximab suggested that those who showed response generally had some degree of steroid sensitivity at the time they received treatment. A previous double-blind, randomised, placebo-controlled trial in children with FR-SSNS and SDNS has shown a significantly longer relapse-free period after treatment with rituximab compared with placebo [206]. Evidence of efficacy of rituximab in these groups of patients with steroid-responsiveness has also derived from several retrospective cohort studies and case series [207]. In contrast, in 31 patients with steroid-resistant and calcineurin inhibitor-resistant nephrotic syndrome, an open-label randomised trial of addition of rituximab did not show reduction in proteinuria at 3 months [193]. The authors did, however, report a non-significant reduction in proteinuria by 48% through addition of rituximab in a subgroup of patients with "delayed resistance" defined as development of resistance to steroids and calcineurin inhibitors months or years after initial diagnosis with NS. A systematic review of rituximab treatment for childhood SRNS included 8 studies and 226 patients [273]. Limited genetic testing was performed in 42 patients and 5 were found to have pathogenic variants in *NPHS2* or *WT1*. The systematic review reported complete or partial remission in 42% of 100 patients with initial steroid-resistant NS and 53.8% of 65 patients

with late steroid-resistant NS. The current study found some response (either CR or PR) in 34.6% of patients with NGPPSR and in 75.0% with SSR.

As discussed in Chapter 2 and previously published [274], SSR was associated with a higher risk of post-transplant disease recurrence and may be a marker of (a) pathogenic circulating factor(s). The efficacy of rituximab in this group of patients may be via an indirect effect on this factor or its downstream mechanisms. It is known that rituximab targets CD20 on B cells and reduces their number and antibody production in addition to affecting T cell proliferation and modulating T-cell subsets [275].

3.4.1.3 Biopsy findings

For the 162 patients who received IIS and with biopsy results available, this study found no significant difference in response to first treatment or all treatments based on initial histology. This suggests that biopsy findings are unlikely to be helpful in making a clinical decision about specific IIS medications.

3.4.1.4 Long term outcomes

This study has shown that the type of response to first IIS treatment is highly significantly associated with progression to ESRF and need for transplantation. The five-year ESRF-free survival rates were 93.8%, 90.3% and 60.0% for patients with CR, PR and no response respectively. These findings suggest that "response to first IIS treatment" has the potential to be used to stratify patients into prognostic groups and may help clinicians decide about subsequent treatments.

Figure 3.12 shows a flow chart which builds on stratification of patients by pattern of steroid resistance and genetics as discussed in Chapter 2 (Figure 2.6) and includes response to first IIS.

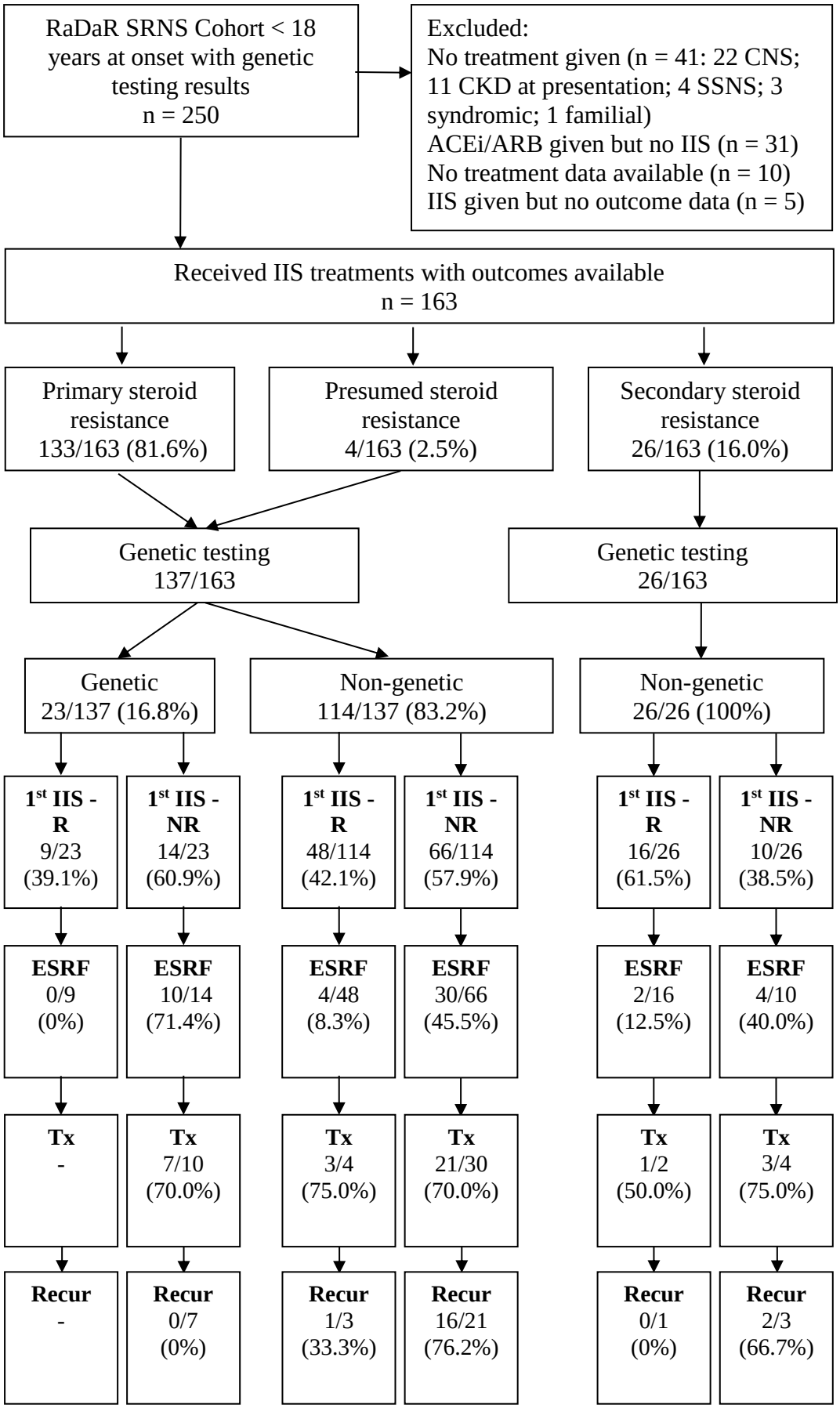
RaDaR SRNS Cohort < 18 years at onset with genetic testing results
n = 250
Excluded:
No treatment given (n = 41: 22 CNS; 11 CKD at presentation; 4 SSNS; 3 syndromic; 1 familial)
ACEi/ARB given but no IIS (n = 31)
No treatment data available (n = 10)
IIS given but no outcome data (n = 5)
Received IIS treatments with outcomes available
n = 163
Primary steroid resistance
133/163 (81.6%)
Presumed steroid resistance
4/163 (2.5%)
Secondary steroid resistance
26/163 (16.0%)
Genetic testing
137/163
Genetic testing
26/163
Genetic
23/137 (16.8%)
Non-genetic
114/137 (83.2%)
Non-genetic
26/26 (100%)
1st IIS - R
9/23
(39.1%)
1st IIS - NR
14/23
(60.9%)
1st IIS - R
48/114
(42.1%)
1st IIS - NR
66/114
(57.9%)
1st IIS - R
16/26
(61.5%)
1st IIS - NR
10/26
(38.5%)
ESRF
0/9
(0%)
ESRF
10/14
(71.4%)
ESRF
4/48
(8.3%)
ESRF
30/66
(45.5%)
ESRF
2/16
(12.5%)
ESRF
4/10
(40.0%)
Tx
-
Tx
7/10
(70.0%)
Tx
3/4
(75.0%)
Tx
21/30
(70.0%)
Tx
1/2
(50.0%)
Tx
3/4
(75.0%)
Recur
-
Recur
0/7
(0%)
Recur
1/3
(33.3%)
Recur
16/21
(76.2%)
Recur
0/1
(0%)
Recur
2/3
(66.7%)

Figure 3.12: Flow chart showing long term outcomes in patients stratified by pattern of steroid resistance, genetics and response to first-line intensified immunosuppressive treatment

Legend: ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CNS, congenital nephrotic syndrome; CKD, chronic kidney disease; ESRF, end-stage renal failure; IIS, intensified immunosuppressive treatment; NR, no response; R, complete or partial response; Recur, post-transplant disease recurrence; SRNS, steroid-resistant nephrotic syndrome; SSNS, steroid-sensitive nephrotic syndrome; Tx, transplanted

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Figure 3.12 illustrates that in all cases, some response to first IIS was associated with a lower rate of progression to ESRF compared with patients who showed no response. This was also the case in patients with a likely-genetic cause for disease: none of the 9 patients who showed some response to IIS had reached ESRF (see Table 3.7 and Table 3.8 for further patient details). It should be noted, however, that for this subgroup of patients the median duration of follow-up was 2.5 years, therefore renal survival at 5 and 10 years is not yet known. At the most recent follow-up, 1 patient was at CKD stage 4, 2 patients at stage 2 and the remaining 6 at stage 1.

Response to first IIS also has potential to help predict post-transplantation recurrence. In the subgroup of patients with non-genetic disease, recurrence occurred in 25% of patients with any response versus 75% with no response, although this difference was not statistically significant ($p = 0.084$). This was probably due to the small numbers of patients who did respond to first IIS that needed transplantation ($n = 4$).

3.4.2 Comparison with other studies

The current study was the first to have examined response to IIS and long-term outcomes in a cohort of children with SRNS, all of whom had had genetic

testing with WES or an extensive gene panel. The most comparable studies were the German cohort of 231 patients published in 2016 [138] and the PodoNet cohort of 1354 patients from 2017 [226]. Each had slightly differing definitions of CR and PR as detailed in Chapter 1 (Section 1.5.3). The German cohort study focused only on response to ciclosporin and reported CR, PR and no response rates of 60.5%, 18.5% and 21.0% respectively in patients with non-genetic disease, and 3.1%, 15.6% and 81.3% respectively in patients with genetic disease. The comparable figures for the current study were 29.9% for CR, 25.3% for PR and 44.8% for no response in patients with non-genetic disease, and 7.1%, 28.6% and 64.3% respectively in those with genetic disease. Although the figures differed, the studies indicated that around 20% or more patients with genetic disease showed some response to ciclosporin. This may have been via its non-immunomodulatory effects directly on the podocyte cytoskeleton [276, 277]. The PodoNet cohort study reported transient CR in 2.7% and PR in 11% of 74 children with genetic disease. Most of these were receiving ciclosporin either alone or in combination with methylprednisolone or ACEi/ARB. Bensimhon *et al*. collated data from 12 studies to identify patients with genetic SRNS who responded to ciclosporin [276]. They reported 10 patients achieving CR and 27 PR. The patients with CR had pathogenic variants in *WT1* (4 patients), *ACTN4* (2), *NPHS1* (2), *NPHS2* (1) and *PLCE1* (1). Those with PR had variants in *NPHS2* (15), *WT1* (10), *COQ6* (1) and *EMP2* (1). These findings together with data from this study suggest that patients with genetic disease with variants in a wide range of genes can respond to IIS. Further study will be required to understand at a functional level how specific pathogenic variants may render the disease responsive to IIS treatments. In the future, if genetic testing was

undertaken at the point of diagnosis of SRNS with results available in a relatively short time-frame, clinicians may be able to use knowledge of specific variants to decide on treatments. The use of clinical genetic testing in SRNS will be the focus of Chapter 4.

Looking at the response to IIS other than ciclosporin, this study showed a significantly lower response rate to cyclophosphamide. This was in keeping with other large cohort studies [226] and a systematic review of treatments for SRNS [192] supporting the recommendation that cyclophosphamide should not be used. The present study showed no difference in response between ciclosporin and tacrolimus. Similar results were seen in an RCT with follow-up to 12 months [191]. However, another prospective comparative study reported that the estimated renal survival at 5 years was significantly better for those treated with tacrolimus (79% versus 33%) with fewer side effects [278].

In the current study, rituximab was associated with CR in 33.3% overall and 66.7% in the subgroup with SSR. Although an open-label RCT showed no benefit of addition of rituximab to prednisolone and a calcineurin inhibitor [193], several smaller studies have suggested benefit. A systematic review including 226 patients with SRNS treated with rituximab reported remission in 40.8% of patients with initial steroid-resistance and 52.8% with late steroid-resistance [273]. The higher apparent CR rate in the SSR subgroup reported here may have been related to some of the patients having a degree of steroid -responsiveness at the time they received rituximab.

Regarding long-term progression to ESRF, findings of this study were generally in agreement with those previously published. Ten-year renal survival rates in this cohort were 93.8%, 81.3% and 25.7% for patients achieving CR, PR

and no response respectively. The equivalent figure for the PodoNet cohort were 94%, 72% and 43% [226]. Ten-year outcomes were not reported in the German cohort study but maintenance of normal renal function at most-recent follow-up (median 94 months) was seen in 98% of patients with CR to ciclosporin, 67% with PR and 47% with no response [138].

3.4.3 Limitations

As a retrospective, non-controlled study, various factors should be considered in the interpretation of the findings. Many patients likely received concomitant treatments alongside IIS. In some cases, IIS were given simultaneously, ACEi/ARB were used with IIS or albumin infusions administered. When two IIS were started in parallel, the outcome during the next 6 months was assigned to both drugs. The use of other therapies alongside IIS were likely to inflate the numbers appearing to show response, particularly for PR defined as an increase in plasma albumin > 25 g/L. Due to the nature of a retrospective study where treatment decisions were made by local clinicians, the direct effects attributable to IIS could not be known with certainty.

All efforts were made to obtain complete data with direct requests for specific data items missing from RaDaR sent to the local teams. Despite this, some patients had no record of medications administered and in other cases the laboratory data available was insufficient to be certain of response to treatment. In the latter case, free text entries, which included anonymised copies of clinic letters, were reviewed and clinicians contacted directly to provide an opinion on the response to treatment using the same definitions of CR and PR.

The outcome data relating to patients with genetic disease were representative only of the subset of patients to whom clinicians decided to give IIS. In most cases it was unlikely that genetic testing results were available when decisions were made about starting the first IIS, therefore baseline clinical characteristics, such as age of onset < 3 months or associated syndromic features, would have influenced that decision. A prospective study with genetic testing at presentation and results available within weeks together with standardised follow-up data would help in the understanding of which patients with genetic disease may benefit.

The analyses using data from all IIS treatments during the course of patients' disease may have been subject to bias resulting from the sequence in which clinicians tend to prescribe the drugs. Those used more frequently as second- or third-line IIS were more likely to be administered to patients with a "drug-resistant" phenotype and so may appear less effective. In order to avoid this bias, the study conducted a separate analysis including only the first-administered IIS.

3.4.4 Conclusion

This study has demonstrated a similar efficacy of tacrolimus and ciclosporin when used a first-line IIS, and significantly higher than cyclophosphamide, but with CR in only around 25%. Rituximab was associated with a better response in patients with SSR compared with those with NGPPSR. Complete response to first IIS was significantly lower in patients with genetic compared with non-genetic disease. However, several patients with a likely-genetic cause for disease did show at least partial response. Irrespective of the genetic aetiology, complete or partial

response to the first-line IIS was associated with better long-term outcomes and kidney survival at 5 and 10 years.

This study focused on a UK cohort, many of whom had genetic analysis by WES on a research basis. Chapter 4 will examine the utility and outcomes of clinical genetic testing in an international cohort of patients with proteinuric kidney disease.

Chapter 4 Clinical Genetic Testing using an SRNS Gene Panel

4.1 Introduction

As discussed in earlier chapters, genetic analysis of patients with SRNS has the potential to stratify patients and help decisions regarding treatments and prognosis. Pathogenic variants in single genes affecting podocyte function are a common cause of SRNS, reported in up to 29.5% of a predominantly childhood cohort [57]. There is a differing spectrum of disease genes and pathogenic variants associated with congenital and childhood-onset disease in comparison with adult disease, and genes may present a renal-only phenotype or NS as part of a wider syndrome. Alport syndrome (AS) is associated with pathogenic variants in *COL4A3, COL4A4 or COL4A5* and may present with proteinuria (some with FSGS on biopsy), and more commonly haematuria [279]. The renal histology is characterised by an alteration of the glomerular basement membrane. Variants in *COL4A3* and *COL4A4* have been identified in association with FSGS together with thin basement membrane nephropathy but without the extra-renal features of AS [280].

The proportion of single gene cases identified inversely correlates with the age of onset with 69.4% - 100% of congenital-onset disease reported as having a genetic aetiology [57, 59]. Over 53 genes, with pathological variants inherited in both recessive and dominant patterns, have been implicated in SRNS [1, 85].

Timely genetic testing can considerably alter patient management, and facilitate a greater understanding of the genetic complexity of the condition [281].

Recent studies report a multiple-gene testing approach using next generation sequencing (NGS) [57, 68, 137, 282]. Only a single NGS study has reported copy number variation in NS genes,[1] hence the contribution of this mechanism to SRNS is currently largely unknown, although evidence has been provided for this as a mechanism [283, 284]. Several studies report patients with a typical phenotype and only a single recessive pathogenic variant [285, 286]. This suggests there may be as-yet uncharacterised variants and that a comprehensive NGS assay with the ability to detect copy number variants (CNVs) would be of increased value.

Collaboration between the University of Bristol and Bristol Genetics Laboratory led to the development of a clinically-approved gene panel test for 37 SRNS and collagen-related genes (Table 4.1) using a targeted amplicon based NGS assay and bespoke bioinformatics analysis that detects both single nucleotide variants (SNVs) and CNVs in batches of 12-16 patients. Importantly, the panel has the flexibility to be extended according to discovery of new genes. An enlarged panel of 70 renal-associated genes has been offered since March 2017 for new referrals including novel genes recently reported associated with SRNS such as *NUP93*, *NUP107*, *NUP205*, *KANK1*, *KANK4*, *MAGI2*, *EMP2* and *ANLN* [89, 96, 107, 108, 112].

The aim of this study was to examine the utility and detection rate of clinical genetic testing and gain an idea of the impact of results on clinical management.

Table 4.1: Genes included in the diagnostic 37-gene panel and coverage

Gene	Chromosome	Exons	Size of target (Kb), Percentage coverage	Inheritance pattern or association	Accession no	Disease association	Key reference
ACTN4 *	19	21	4.2, 99.8%	AD	NM_004924	Familial and sporadic SRNS (usually adult)	[63]
ALG1	16	13	2.0, 90.5%	AR	NM_019109	Congenital disorder of glycosylation	[131]
ALMS1	2	23	13.7, 98.9%	AR	NM_015120	Alström syndrome, retinitis pigmentosa, sensorineural hearing loss	[134]
APOL1 *	2	7	1.9, 97.9%	Risk factor	NM_145343	Increased susceptibility to FSGS in African Americans and those of African ancestry	[101]
ARHGAP24 *	4	10	2.9, 99.2%	AD	NM_001025616	FSGS	[104]
ARHGDIA	17	6	1.4, 100%	AR	NM_001185077	CNS	[105]
CD151	11	9	1.1, 100%	AR	NM_004357	NS, pretibial bullous skin lesions, neurosensory deafness, bilateral lacrimal duct stenosis, nail dystrophy, and thalassemia minor	[130]
CD2AP *	6	18	2.8, 99.9%	AD/AR	NM_012120	FSGS / SRNS	[87]
COL4A3	2	52	8.3, 98.4%	AR	NM_000091	Alport syndrome	[83, 287]
COL4A4	2	48	7.5, 99.8%	AR	NM_000092	Alport syndrome	[83, 287]
COL4A5	X	53	7.9, 99.1%	X-linked	NM_033380	Alport syndrome	[287]
COQ2 *	4	7	1.7, 100%	AR	NM_015697	Mitochondrial disease, encephalopathy / isolated nephropathy	[118]
COQ6 *	14	12	2.3, 100%	AR	NM_182476	NS +/- sensorineural deafness; DMS	[119]
COQ7	16	6	1.1, 100%	AR	NM_016138	Mitochondrial disease, encephalopathy	[120]
COQ9	16	9	1.5, 99.8%	AR	NM_020312	Mitochondrial disease, encephalopathy, renal tubulopathy	[121]
CYP11B2	8	9	2.0, 97.0%	Association	NM_000498	Corticosterone methyloxidase deficiency, Familial hyperaldosteronism	[125]

E2F3	6	7	1.8, 99.5%	AD	NM_001949	FSGS + mental retardation (whole gene deletion)	[92]
INF2 *	14	23	5.2, 97.8%	AD	NM_022489	Familial and sporadic SRNS, FSGS-associated Charcot-Marie-Tooth disease	[61]
ITGA3	17	26	4.8, 98.6%	AR	NM_002204	Interstitial lung disease, CNS, and mild epidermolysis bullosa	[126]
ITGB4	17	40	7.8, 99.4%	AR	NM_000213	Epidermolysis bullosa and pyloric atresia, FSGS	[127]
KANK2	19	11	3.1, 100%	AR	NM_015493	SSNS/SDNS +/- haematuria	[112]
LAMB2 *	3	32	7.0, 100%	AR	NM_002292	Pierson syndrome	[12]
LMX1B *	9	8	1.6, 99.4%	AD	NM_002316	Nail patella syndrome; also FSGS without extrarenal involvement	[64]
MED28	4	4	0.8, 100%	AR	NM_025205	NS	[106]
MYH9	22	41	8.2, 100%	AD, association	NM_002473	MYH9-related disease; Epstein and Fechtner syndromes	[98]
MYO1E *	15	28	5.0, 99.9%	AR	NM_004998	Familial SRNS	[102, 103]
NPHS1 *	19	29	5.2, 99.9%	AR	NM_004646	CNS / SRNS	[55]
NPHS2 *	1	8	1.6, 100%	AR	NM_014625	CNS / SRNS	[56]
PDSS2	6	8	1.9, 99.4%	AR	NM_020381	Leigh syndrome	[123]
PLCE1 *	10	33	8.9, 99.7%	AR	NM_016341	CNS / SRNS	[60]
PMM2	16	8	1.4, 100%	AR	NM_000303	Congenital disorder of glycosylation	[132]
PTPRO *	12	27	5.0, 99.7%	AR	NM_030667	NS	[88]
SCARB2	4	12	2.1, 100%	AR	NM_005506	Action myoclonus-renal failure syndrome +/- hearing loss	[133]
SMARCAL1	2	18	3.8, 99.9%	AR	NM_014140	Schimke immuno-osseous dysplasia	[91]
TRPC6 *	11	13	3.4, 98.8%	AD	NM_004621	Familial and sporadic SRNS (mainly adults)	[62]
WT1 *	11	10	2.1, 99.1%	AD	NM_024426_449AAs.3	Sporadic SRNS (children—may be associated with abnormal genitalia); Denys-Drash and Frasier syndrome	[65]
ZMPSTE24	1	10	1.9, 100%	AR	NM_005857	Mandibuloacral dysplasia with FSGS	[122]

* indicates genes included in the initial 16 gene panel

Legend: AD, autosomal dominant; AR, autosomal recessive; CNS, congenital nephrotic syndrome; DMS, diffuse mesangial sclerosis; FSGS, focal segmental glomerulosclerosis; Kb, kilobases; NS, nephrotic syndrome; SDNS, steroid-dependent nephrotic syndrome; SRNS, steroid-resistant nephrotic syndrome; SSNS, steroid-sensitive nephrotic syndrome

4.2 Methods

4.2.1 Patient Cohort

302 patients were referred with informed consent for diagnostic gene panel analysis via Consultant Nephrologists and Consultant Clinical Geneticists over a 26-month period with clinical data supplied by clinical proforma. The diagnostic test has been formally assessed for validity, and socio-legal/ethical implications by the UK Genetic Testing Network and UK NHS commissioners through the gene dossier process, and was undertaken in an accredited UK NHS Laboratory. Data presented pertains only to anonymised auditing of routine diagnostic testing; therefore, this study was not subject to ethical approval.

4.2.2 Assay Design, Target Enrichment and Sequencing

All genetic sequencing and analysis were undertaken by staff at Bristol Genetics Laboratory. A custom HaloPlex Target Enrichment System (Agilent) was designed to target 37 genes (exons and 25bp of flanking intron) associated with SRNS. DNA was prepared from venous blood samples using an Autopure Gentra system (Qiagen) or referred as DNA from external laboratories. Genomic DNA (225ng) was processed for each sample according to the manufacturer's protocol. Library QC was performed using an Agilent TapeStation 2200. Samples were pooled in typical batches of 12-16 and sequenced using 2x150bp paired end sequencing on a MiSeq (Illumina) analyser following manufacturer's protocol. Twenty-two patients were sequenced on an earlier version of the panel comprising 16 genes as indicated in Table 4.1. After initial analysis, gap filling by Sanger sequencing was undertaken on the rare occasions where a gap in coverage was

found in a clinically-relevant gene or where a single LP variant was detected in a recessive gene.

4.2.3 Bioinformatic Analysis

Analysis was performed using a bespoke pipeline based on the Broad Institute's Best Practice guidelines [288, 289]. FASTQs were hard trimmed to remove HaloPlex adapter sequences and read through, the trimmed reads were then mapped to UCSC GRCh37/hg19 FASTA reference using BWA-MEM. GATK (version 1.6) unified genotyper was used for indel realignment and variant calling, with quality, capture and alignment metrics generated using Picard. Pindel was used for additional long insertion/deletion and structural variant detection. Variants were annotated and stratified for analysis using Geneticist Assistant (SoftGenetics Version 1.1.5 Release Build 189 Revision 6848).

4.2.4 Variant Classification

Variants were classified according to the Association for Clinical Genetic Science best practice guidelines for the evaluation of pathogenicity and reporting of sequence variants: Class 1 - clearly not pathogenic, Class 2 - unlikely to be pathogenic, Class 3 - unknown significance, Class 4 - likely to be pathogenic and Class 5 - clearly pathogenic [290]. Variants were assessed using Alamut software v2.3.1 (Interactive Biosoftware, Rouen, France). Classification considered literature evidence, disease mechanism and phenotype, evolutionary conservation including relevant functional domains and population frequency (NHLBI Exome Variant Server, dbSNP and ExAC [total allele frequency]) (Table 4.2). Variants with a frequency >1% in any population were excluded from further investigation

(with the exception of the *NPHS2* p.(Arg229Gln) variant). In addition, web-based prediction tools PolyPhen-2, Align GVGD and SIFT were used for the assessment of missense variants and splice site variants were investigated with prediction programs SpliceSiteFinder, MaxEntScan, Human Splice Finder, NNSPLICE and GeneSplicer. Class 3, 4 and 5 variants were confirmed by Sanger sequencing (BigDye Terminator v3.1 Cycle Sequencing Kit and ABI 3730 Applied Biosystems).

Table 4.2: Variant classification criteria

Class 5: Pathogenic	Reported in the literature as pathogenic supported by functional evidence **OR** segregation studies **OR** multiple independent case reports **AND** Consistent with phenotype of patient, inheritance and disease mechanism
Class 4: Likely pathogenic	Minor Allele Frequency (MAF) <1%* **AND** Not reported or literature evidence sparse**,** with no segregation studies or functional analysis available **AND** Consistent with phenotype of patient, inheritance and disease mechanism ***AND*** - Missense variant in functional domain with high conservation and supporting *in silico* results **OR** - Nonsense or frame shift variant **OR** - Invariant splice site (+/-2) variant or highly conserved synonymous variant with >3/5 *in silico* splice prediction tools returning a >10% difference in splice site prediction value between reference sequence and variant.
Class 3: Unknown Significance (VUS)	MAF <1% **AND** - Inconclusive or conflicting *in silico* results, not reported in the literature, but consistent with phenotype, inheritance and disease mechanism OR - *In silico* predictions class the variant as Class 4, but not consistent with phenotype of patient
Class 2: Likely Benign	MAF <1% **AND** - *In silico* results indicate weak amino acid conservation and a benign impact on protein **OR** - Synonymous or intronic change in a weakly conserved nucleotide with no *in silico* effect on splicing **OR**

	- Inconclusive or conflicting *in silico* results, not reported in the literature, not consistent with phenotype of patient, inheritance, or disease mechanism **OR** - Sparse literature evidence indicating benign status **OR** - Limited segregation studies not supporting pathogenicity
Class 1: Benign	MAF >1% **OR** MAF<1% and proven as non-pathogenic in published literature
* <1% MAF in any population in EVS, dbSNP, 1000 genomes and ExAC databases; exception of non-neutral polymorphism *NPHS2*, c.686G>A p.(Arg229Gln).	

4.2.5 Variant Segregation Analysis

Analysis of parental samples and those from other available affected/unaffected relatives was undertaken where possible using Sanger sequencing to determine phase (*cis* or *trans*) and to gather evidence supporting pathogenicity by genotype/phenotype concordance in the family.

4.2.6 Copy Number Analysis

Copy number variants (CNVs) were identified by CONTRA using log-ratios of GC corrected, library balanced, binned and interpolated read depth data [291]. CNVs were confirmed using Multiplex Ligation-dependent Probe Amplification (MLPA) with custom designed probes and the MRC-Holland P200-A1 Human DNA Reference-1 probe mix following the manufacturer's protocol.

4.3 Results

4.3.1 Source of referrals

Referrals were received from 12 different countries (Figure 4.1).

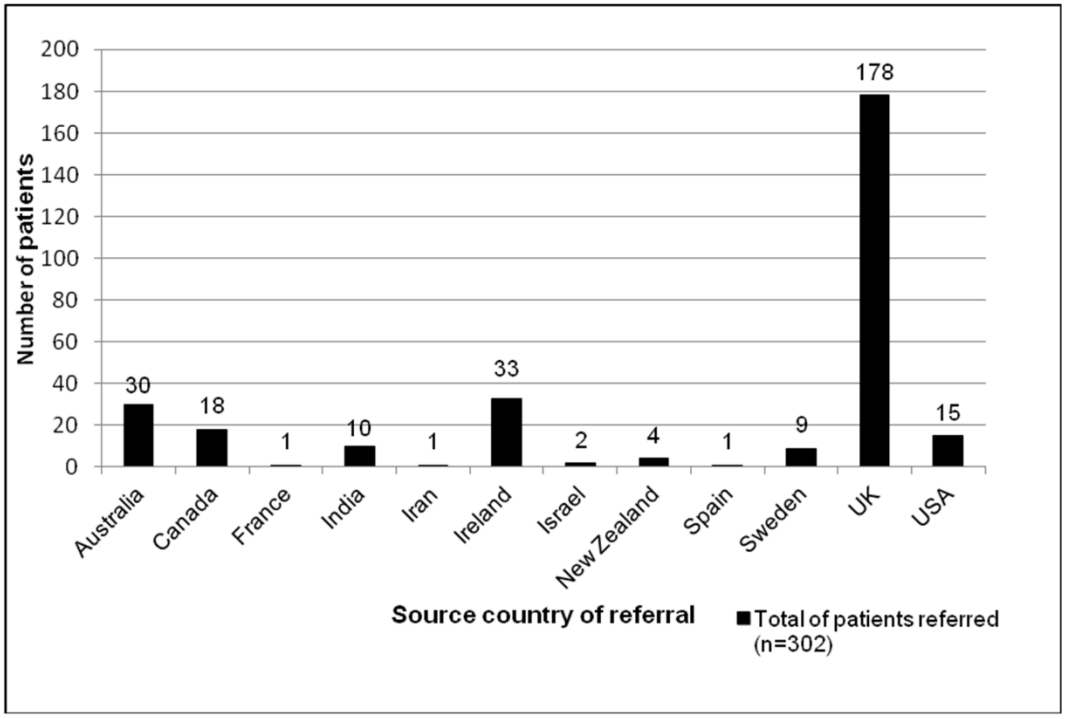

Figure 4.1: Source of referrals for NGS gene panel testing

4.3.2 Demographics

The majority of patients had a clinical diagnosis of idiopathic nephrotic syndrome, mostly SRNS, but 12 were steroid sensitive (SSNS) either frequently-relapsing or steroid-dependent. Thirty-five patients were referred with features suggestive of AS including haematuria, a family history, hearing loss or thin basement membrane on biopsy. For clinical analyses, the cohort was, therefore, separated into SRNS, SSNS and Alport groups. The timing of disease onset was known for 196 patients: 32 (16.3%) were congenital (< 3 months), 16 (8.2%) infantile (3-12 months), 101 (51.5%) childhood (1-12 years), 17 (8.7%) juvenile (13-18 years) and 30 (15.3%) adult (>18 years). Of 255 patients with SRNS, a biopsy report was available in 133 which showed FSGS in 109 (82.0%) and minimal change disease in 8 (6.0%). In 9 of 12 SSNS patients with a biopsy report, 3 (33.3%) had FSGS and 3 (33.3%) had MCD. In patients with SRNS, 35 (23.8%) of 147 with

data available had a family history of renal disease. Among 132 SRNS patients where data on age of onset and family history were available, 52.6% (10/19) of adults compared with 12.4% (14/113) of patients <18 years had a positive family history. This may represent differing referral patterns in clinicians caring for adult patients with NS such that they were less likely to request genetic testing for adults without a family history. Among 35 patients in the Alport group, 78.6% of 28 patients with data had a family history of a similar disease. Other demographic data are shown in Table 4.3.

Table 4.3: Clinical characteristics of the cohort

		Total cohort	Steroid-resistant nephrotic syndrome	Steroid-sensitive nephrotic syndrome	Haematuria / Alport syndrome
Total patients		302	255	12	35
Male (%)		165 (54.6)	138 (54.1)	10 (83.3)	17 (48.6)
Age at onset / testing* in years (%)	0-0.25	32 (10.6)	31 (12.2)	1 (8.3)	0 (0)
	0.25-1	16 (5.3)	13 (5.1)	0 (0)	3 (8.6)
	1-12	147 (48.7)	125 (49.0)	9 (75)	13 (37.1)
	13-18	45 (14.9)	40 (15.7)	1 (8.3)	4 (11.4)
	> 18	62 (20.5)	46 (18.0)	1 (8.3)	15 (42.9)
Family history positive / number with data available (%)		58 / 183 (31.7)	35 / 147 (23.8)	1 / 8 (12.5)	22 / 28 (78.6)
Consanguinity / number with data available (%)		17 / 141 (12.1)	13 / 117 (11.1)	3 / 9 (33.3)	1 / 15 (6.7)
Ethnicity (% of patients where data available)	White	99 (65.8)	83 (66.4)	3 (37.5)	13 (76.5)
	Indian	14 (9.4)	12 (9.6)	2 (25)	0
	Black African / Caribbean	7 (4.7)	5 (4.0)	1 (12.5)	1 (5.9)
	Pakistani	6 (4.0)	3 (2.4)	2 (25)	1 (5.9)
	Bangladeshi	2 (1.3)	2 (1.6)	0 (0)	0 (0)
	Asian	2 (1.3)	2 (1.6)	0 (0)	0 (0)
	Middle Eastern	2 (1.3)	2 (1.6)	0 (0)	0 (0)
	Arabic	2 (1.3)	2 (1.6)	0 (0)	0 (0)

	Mixed	3 (2.0)	3 (2.4)	0 (0)	0 (0)
	Other	13 (8.7)	11 (8.8)	0 (0)	2 (11.8)
	No ethnicity data available	152	130	4	18
Biopsy findings (% of patients where data available); number (%) with genetic diagnosis	FSGS	115 (71.9); 27 (23.5)	109 (82.0); 26 (23.9)	3 (33.3); 0 (0)	3 (16.7); 1 (33.3)
	MCD	11 (6.9); 0 (0)	8 (6.0); 0 (0)	3 (33.3); 0 (0)	0 (0)
	Mesangioproliferative GN	3 (1.9); 0 (0)	3 (2.3); 0 (0)	0 (0)	0 (0)
	DMS	5 (3.1); 2 (40.0)	4 (3.0); 2 (50.0)	1 (11.1); 0 (0)	0 (0)
	Finnish type	2 (1.3); 2 (100)	2 (1.5); 2 (100)	0 (0)	0 (0)
	Alport	8 (5.0); 7 (87.5)	0 (0)	0 (0)	8 (44.4); 7 (87.5)
	TBMN	4 (2.5); 1 (25.0)	0 (0)	0 (0)	4 (22.2); 1 (25.0)
	Other	12 (7.5); 3 (25.0)	8 (6.0); 3 (37.5)	2 (22.2); 0 (0)	3 (16.7); 0 (0)
	No biopsy data available / Not biopsied	142	122	3	17
Total with likely-pathogenic variants (%)		71 (23.5)	54 (21.2)	0 (0)	17 (48.6)

* For patients where no data were available for age at disease onset, the age at genetic testing was used.
Legend: DMS, diffuse mesangial sclerosis; FSGS, focal segmental glomerulosclerosis; GN, glomerulonephritis; MCD, minimal change disease; TBMN, thin basement membrane nephropathy

4.3.3 Data quality and gene panel performance

An average gene coverage of 99.26% coding sequence at a minimum read depth of 30x was achieved on a typical 12-patient run. The per gene coverage is shown in Table 4.1. The ACGS reporting time guideline for large panel tests is 112 calendar days [292]. The median time from receipt of samples to issue of a report was 74 days (interquartile range: 49-106 days). With clinically urgent referrals, it was possible to reduce substantially the turnaround time with complete genetic panel reports provided for 17 patients within 4 weeks and the fastest positive case report (*NPHS2* compound heterozygote) being issued in 22 calendar days.

4.3.4 Genetic variants

Targeted gene panel testing of all 302 patients identified 71 (23.5%) with a likely genetic cause for disease. The genetic diagnostic rate among the group with SRNS was 21.2%, including 44/209 (21.1%) paediatric and 10/46 (21.7%) adult nephrotic cases (Figure 4.2). In SRNS patients where family history was known, the genetic diagnostic rate was 11/35 (31.4%) in those with a positive family history and 30/112 (26.8%) in those with negative family history. In SRNS patients where family history was known and with age of onset over 18 years, the genetic diagnostic rate was 2/10 (20.0%) in those with a positive family history and 3/9 (33.3%) in those with negative family history. The rate in those with parental consanguinity was 5/13 (38.5%) compared with 28/104 (26.9%) in those without. In all the 12 SSNS patients, no pathogenic variants were found in any of the 37 genes tested. The genetic diagnostic rate was 48.6% for the Alport group.

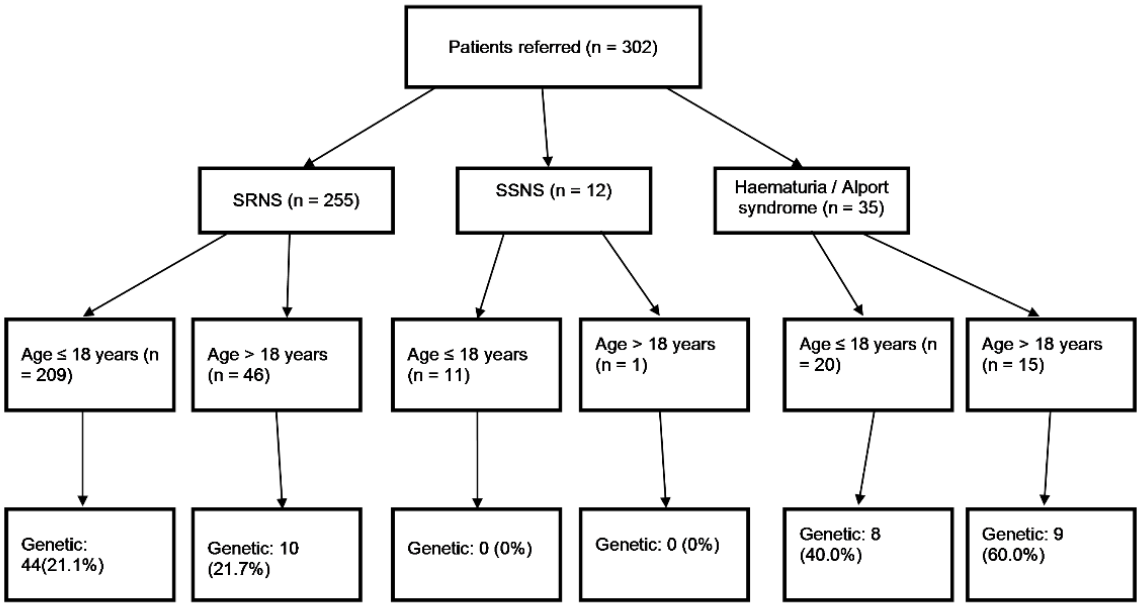

Figure 4.2: Flow chart of patients by phenotype at presentation, age and genetic diagnosis

"Age" refers to age at diagnosis or, if not available, age at genetic testing. "Genetic" refers to the number of cases with likely-pathogenic variants. SRNS, steroid-resistant nephrotic syndrome; SSNS, steroid-sensitive nephrotic syndrome.

The spectrum of pathogenic variants is summarised in Table 4.4. Detailed phenotypic and variant data for the 71 patients with genetic disease are shown in Table 10.3 in the Appendices.

Table 4.4: Genes with likely-pathogenic variants in the steroid-resistant nephrotic syndrome group and haematuria / Alport syndrome group

Gene with likely-pathogenic variant	**Steroid-resistant nephrotic syndrome** (number of patients)			**Haematuria / Alport syndrome** (number of patients)		
Age group *	≤ 18 years (n = 209)	> 18 years (n = 46)	Total (n = 255)	≤ 18 years (n = 20)	> 18 years (n = 15)	Total (n = 35)
NPHS1	12	0	12	0	0	0
WT1	9	2	11	0	0	0
NPHS2	7	0	7	0	0	0
LMX1B	4	0	4	0	0	0
INF2	0	3	3	0	0	0
LAMB2	3	0	3	0	0	0
MYH9	2	0	2	0	0	0
PLCE1	2	0	2	0	0	0
ACTN4	1	0	1	0	0	0
SCARB2	0	1	1	0	0	0
SMARCAL1	1	0	1	0	0	0
TRPC6	0	1	1	0	0	0
COL4A3	0	1	1	3	2	5
COL4A4	1	1†	2	0	2	2
COL4A5	2	1	3	5	5	10
Total	44	10	54	8	9	17

***** For patients where no data were available for age at disease onset, the age at genetic testing was used. None of the 12 patients with steroid-sensitive nephrotic syndrome had likely-pathogenic variants and so are not shown in this table. † This patient was referred with hypertensive nephrosclerosis and a family history of renal disease.

The most frequently-detected LP variants in the SRNS group (n = 255) were in *NPHS1*, *WT1* and *NPHS2* in 12 (4.7%), 11 (4.3%) and 7 (2.7%) patients respectively. In the Alport group, the variants were all in collagen genes: *COL4A3*, *COL4A4* and *COL4A5* in 5 (14.3%), 2 (5.7%) and 10 (28.6%) patients respectively. Of note, 5 SRNS/FSGS patients had LP variants in *COL4A3*, *COL4A4* and *COL4A5* (1, 1 and 3 patients respectively) including a single novel LP *COL4A3* variant, c.698G>A, p.(Gly233Glu), in a patient with a dominant family history of FSGS (patient 4) which tracked with disease in an affected brother. Among patients with SRNS/FSGS who were found to have genetic disease (excluding those with collagen variants), there was an autosomal dominant mode of inheritance in 16/41 (36.6%) of those with disease onset ≤ 18 years compared with 6/7 (85.7%) of those > 18 years.

4.3.5 Variants of unknown significance (VUS)

In addition to the patients with LP variants, a further 40 patients had one or more VUS (Table 4.5). Among the 52 VUS in these patients, the most frequently-involved genes were: 28.9% in collagen genes; 7.7% each in *NPHS1* and *NPHS2*; and 5.8% each in *INF2*, *MYH9*, *PLCE1* and *PTPRO*. Of the 71 patients with likely-genetic disease, 11 cases had 12 VUS in genes other than the one thought to be causative in that patient. In these cases, 41.7% of VUS were in collagen genes and 16.7% in *WT1*. Overall, of the 64 recorded VUS, 31.3% were in collagen genes, 6.3% in *NPHS2*, 6.3% in *NPHS1* and 6.3% in *MYH9*.

Table 4.5: Patients with variants of unknown significance

Patient number	Sex	Age at onset (years)	Presentation	Ethnicity; FH; Consanguinity	Biopsy	Clinical impact	Gene	Nucleotide; segregation	AA
72	F	53*	SRNS	ND; ND; ND	ND	ND	*ACTN4*	c.2084G>A	p.(Arg695His)
73	M	2	SRNS	In; N; N	MCD	No change	*ACTN4*	c.2629G>A	p.(Glu877Lys)
							PTPRO	c.2117G>A	p.(Cys706Tyr)
74	M	13*	Haematuria, familial microscopic haematuria	ND; Y; ND	ND	ND	*ALG1*	c.1127C>T	p.(Pro376Leu)
							ALG1	c.1187+13C>A	p.(?)
75	M	13	NS, SCD	ND; ND; ND	ND	ND	*CD2AP*	c.1637C>T	p.(Ser546Phe)
76	F	44*	SRNS	ND; Y; ND	FSGS	ND	*COL4A3*	c.1855G>A	p.(Gly619Arg)
77	M	30	SRNS	W; N; N	FSGS	No change	*COL4A3*	c.2155T>C	p.(Ser719Pro)
							COL4A3	c.4664C>T	p.(Ala1555Val)
78	M	19	Alport, FH haematuria and hearing loss	W; Y; N	Other	ND	*COL4A3*	c.2313_2330del; mat; unaffected	p.(Leu775_Gly780del)
79	M	15*	SRNS	ND; ND; ND	ND	ND	*COL4A4*	c.136C>A	p.(Pro46Thr)
80	M	8*	SRNS	ND; ND; ND	ND	ND	*COL4A4*	c.809G>A	p.(Gly270Glu)
81	M	17*	Haematuria and proteinuria	W; Y; ND	Alport	ND	*COL4A4*	c.4291C>T	p.(Arg1431Cys)
82	M	49	SRNS	Iraqi; Y; N	FSGS, laminopathy	Imm not started	*COL4A4*	c.4576A>G	p.(Asn1526Asp)
							COL4A4	c.4810-15_4810-14delTT	p.(?)
83	F	53*	Haematuria	W; Y; N	TBMN	Imm not started	*COL4A5*	c.466-3T>A	p.(?)
							COL4A5	c.3285T>C	p.(=)
84	M	32*	SRNS	ND; Y; Y	ND	ND	*COL4A5*	c.2017A>G	p.(Arg673Gly)
							MYO1E	c.3236A>G	p.(Asp1079Gly)
85	M	24	SRNS	W; Y; N	FSGS	Cessation	*COL4A5*	c.2326G>A; mat, tracks with disease in mother and brother	p.(Asp776Asn)

86	F	2	Steroid dependent NS	Pa; N; Y	MCD	ND	*COL4A5*	c.3691C>T	p.(Pro1231Ser)
87	M	18*	SRNS	ND; ND; ND	ND	ND	*COQ2*	c.286C>T	p.(Pro96Ser)
88	F	50	SRNS	W; Y; N	FSGS	ND	*INF2*	c.763G>T; also in son with proteinuria, absent in unaffected daughter	p.(Asp255Tyr)
							LMX1B	c.115C>A; absent in son with proteinuria, in unaffected mother	p.(Pro39Thr)
89	M	1	SRNS	ND; ND; ND	ND	ND	*PTPRO*	c.1300G>A	p.(Glu434Lys)
90	F	14*	SRNS	W; Y; ND	Not done	Possibly change imm	*INF2*	c.2942G>C	p.(Arg981Thr)
91	F	9*	Haematuria	ND; Y; ND	ND	ND	*LAMB2*	c.1156T>C	p.(Cys386Arg)
92	M	62*	SRNS	ND; ND; ND	MPGN	ND	*LAMB2*	c.3533G>A	p.(Arg1178His)
93	M	2	SRNS	ND; Y; N	ND	ND	*MYH9*	c.1784A>G	p.(Asn595Ser)
94	F	9	SRNS, single kidney	W; Y; N	Not done	Imm not started	*MYH9*	c.3215C>T; also in affected sister	p.(Ala1072Val)
95	F	5.5	SRNS	Ar; N; N	Not done	Variant found was not classified as likely-pathogenic therefore not influenced treatment strategy	*MYH9*	c.3838G>A	p.(Val1280Met)
96	F	3*	SRNS	ND; ND; ND	FSGS	ND	*MYO1E*	c.1547A>G	p.(Asp516Gly)
97	F	2	SRNS	W; N; N	FSGS	ND	*NPHS1*	c.2746G>T; pat	p.(Ala916Ser)
98	F	17*	NS, short stature	ND; ND; ND	ND	ND	*NPHS1*	c.2746G>T	p.(Ala916Ser)
99	M	23	SRNS	Pa; Y; N	FSGS	ND	*NPHS1*	c.3027C>G	p.(Tyr1009*)
100	M	42*	Haematuria, hearing loss	W; Y; N	TBMN	ND	*NPHS2*	c.156delG	p.(Thr53Profs*46)
101	M	22*	SRNS	W; N; N	MPGN	ND	*NPHS1*	c.2591G>A	p.(Arg864His)
102	ND	0	SRNS	ND; ND; ND	ND	ND	*NPHS2*	c.860A>G	p.(Gln287Arg)
103	F	12*	SRNS	Samoan; N; N	ND	ND	*NPHS2*	c.1064A>G	p.(Asn355Ser)
							NPHS2	c.138G>A	p.(=)
							PLCE1	c.3580G>A	p.(Gly1194Arg)
							ARHGAP24	c.1057_1058delinsAA	p.(Ala353Asn)
							COL4A5	c.4309C>G	p.(Gln437Glu)
104	F	11*	SRNS	ND; ND; ND	FSGS	ND	*PLCe1*	c.1478G>A	p.(Arg493Gln)
105	M	18*	SRNS	ND; ND; ND	FSGS	ND	*PLCe1*	c.2032A>G	p.(Met678Val)
106	M	7	SRNS	W; Y; N	ND	ND	*PMM2*	c.24_delC	p.(Cys9Alafs*27),
107	M	3.5	SRNS	W; N; N	FSGS	Increase	*PMM2*	c.691G>A; pat	p.(Val231Met)
108	F	19*	SRNS	ND; ND; ND	FSGS	ND	*PTPRO*	c.1631C>T	p.(Thr544Met)
109	M	14	SRNS	ND; ND; ND	ND	ND	*TRPC6*	c.1A>G	p.(Met1?)
							INF2	c.395G>A	p.(Ser120Asn)
110	F	8*	NS	ND; ND; ND	ND	ND	*TRPC6*	c.2392G>C	p.(Asp798His)
111	M	4	SRNS	W; N; N	Membranous	ND	*WT1*	c.844T>C	p.(Cys282Arg)

* Denotes age at genetic testing where age at disease onset was not available
Segregation analysis to clarify pathogenicity is ongoing in 15 of these patients with VUS.

Legend:

AA, amino acid; Ar, Arabic; CKD, chronic kidney disease; ESRF, end stage renal failure; FH, family history; FSGS, focal segmental glomerulosclerosis; Imm, immunosuppression; In, Indian; mat, maternal; MPGN, membranoproliferative glomerulonephritis; N, no; n/a, not available; ND, not done/no data; NS, nephrotic syndrome; Pa, Pakistani; pat, paternal; SCD, sickle cell disease; SRNS, steroid resistant nephrotic syndrome; TBMD, thin basement membrane disease; W, White; Y, yes

4.3.6 Novel variants

Among the 71 patients with a genetic cause for disease, 32 had variants without a previous disease association including 26 with one or more novel variants absent from population databases. Two patients had gene deletions of one or more exons detected by CNV analysis (Figure 4.3). Patient 44 (see Table 10.3 in the Appendices) presented with congenital nephrotic syndrome (CNS) and had a maternally-inherited truncating deletion of *NPHS1* exons 23-29 together with a paternally-inherited previously-reported nonsense variant c.866G>A p.(Trp289*) [74]. Patient 55 also presented with CNS and genetic testing revealed a maternally-inherited frame shift deletion of *NPHS2* exon 2 in combination with a paternally-inherited c.1032delT variant. The c.1032delT variant has been previously reported as the most frequent pathogenic variant in *NPHS2* in Poland (Kashubian region) [286]. Both parents of patient 55 are of Polish extraction. In addition to these two patients, a further 30 had variants without a previous disease association in the following genes: *ACTN4* (1 patient), *COL4A3* (2), *COL4A4* (1), *COL4A5* (10), *INF2* (2), *LAMB2* (2), *NPHS1* (5), *NPHS2* (2), *SMARCAL1* (1), *TRPC6* (1) and *WT1* (3).

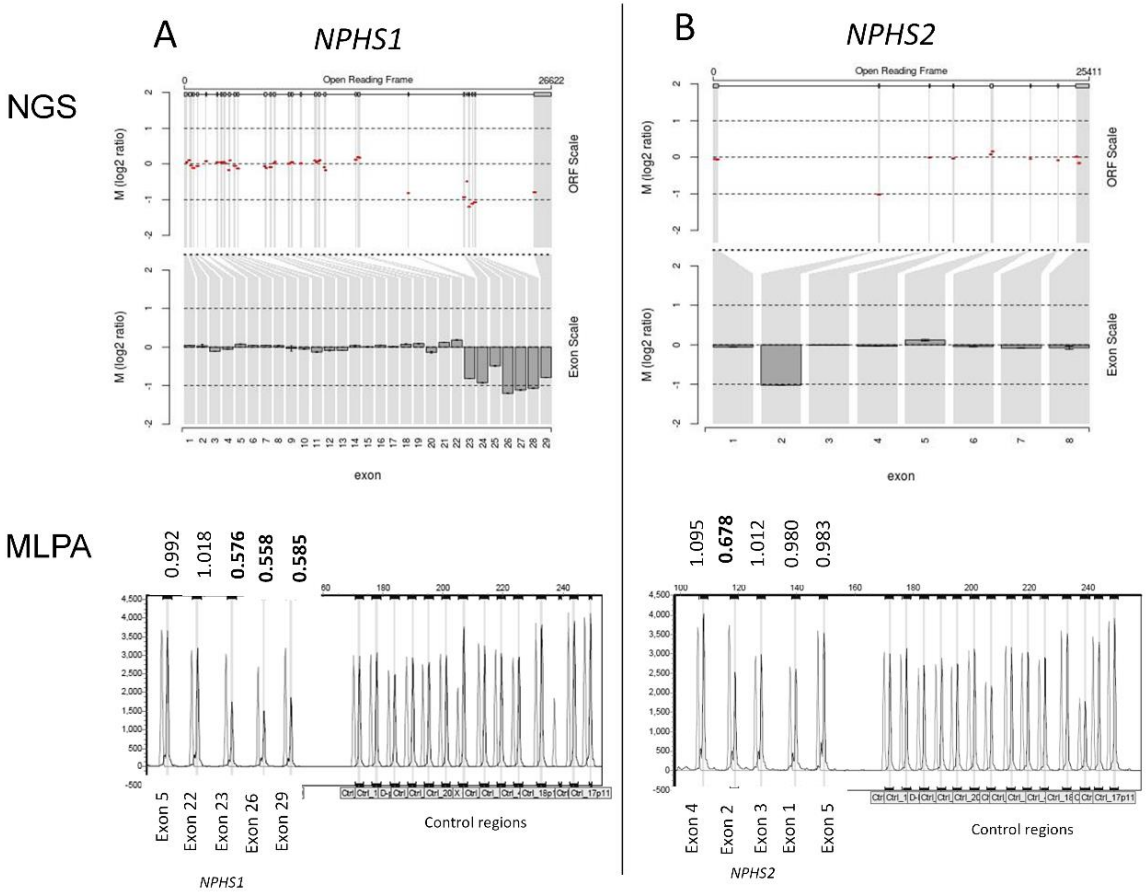

Figure 4.3: *NPHS1* and *NPHS2* Copy number variants

Figure 4.3: *A. NPHS1* deletion of Exon 23-29 B. *NPHS2* deletion of Exon 2. NGS read depth analysis (top) with results confirmed by MLPA (below). NGS fold change in copy number (Log_2 ratio) is shown across the locus (ORF scale) and averaged for each exon (exon scale). MLPA shows patient peaks in dark grey and normal control peaks in light grey. The patient/normal ratios are shown with deletions having ratio < 0.75.

Novel *INF2* LP variants were detected in two adult-onset NS patients: p.(Tyr50Asp) (patient 25) and p.(Leu165Arg) (patient 26). Both were missense variants affecting highly-conserved residues within the diaphanous inhibitory domain (DID) of the INF2 protein consistent with the previously-reported spectrum of disease-causing variants. Segregation supports pathogenicity, p.(Tyr50Asp) co-segregating with disease in 5 affected family members and p.(Leu165Arg) present in one affected family member and absent in 2 unaffected family members. An additional sensory neuropathy phenotype previously reported in 12.5% of *INF2* cases [293] was also seen in affected family members with the p.(Tyr50Asp) variant.

Two novel missense *WT1* variants were identified in the known hotspot region (exons 6-9) [81] in patients with atypical presentation and no recorded extra-renal manifestations. p.(His339Arg) in exon 7 (patient 61) co-segregated with disease in 6 affected family members with a variable phenotype ranging from childhood-onset nephrotic-range proteinuria to mild proteinuria presenting in adulthood. The p.(Arg390Gln) variant in *WT1* exon 8 (patient 65) was associated with age of onset of 30 years and was inherited from an affected father who was diagnosed in his 30s. Although |*WT1* is normally associated with childhood-onset disease, these findings are consistent with a previously-described biphasic childhood and adulthood presentation of variants in *WT1* [57].

4.3.7 Single heterozygous variants in recessive NPHS1 and NPHS2

In six clinically-affected SRNS cases (patients 112-117 in Table 4.6), full coding sequence analysis detected a single heterozygous pathogenic variant in *NPHS1* or *NPHS2* that has been previously published as disease-causing. No CNVs were identified. The finding of these variants may be incidental. However, given the low incidence of SRNS, the young age of onset (below 5 years, two with CNS) and the low frequency/absence of the variants in databases of subjects without known renal disease it is possible that there are additional *NPHS1/NPHS2* variants in unsequenced intronic or promoter regions which may act in combination to cause the phenotype in these patients. These findings were reported as compatible with the phenotype but insufficient to make a diagnosis.

Table 4.6: Genotypes and phenotypes of patients with single heterozygous variants in *NPHS1* and *NPHS2*

Patient number	Sex	Age at onset (years)	Presentation	Ethnicity; FH; Consanguinity	Biopsy	Pathogenicity	Gene	Nucleotide; segregation	AA	Reference	Mutation prediction: SIFT; PolyPhen	Allele frequency: dbSNP; EVS; ExAC
112	M	4	SRNS	W; N; N	MCD	LP	***NPHS1***	c.313G>A	p.(Asp105Asn)	[294]		
113	M	3*	SRNS	ND; ND; ND	ND	LP	***NPHS1***	c.895C>T	p.(Arg299Cys)	[74]		
114	F	0	CNS, clinodactyly 2nd toe	Jordanian; N; Y	FSGS	LP	***NPHS1***	c.1138C>T	p.(Gln380*)	[295]		
115	M	0.3	CNS	W; N; N	Not done	LP	***NPHS2***	c.413G>A; pat	p.(Arg138Gln)	[296]		
						NNP	*NPHS2*	c.686G>A; mat	p.(Arg229Gln)	[69, 297]		
116	F	4*	SRNS	ND; ND; ND	FSGS	LP	***NPHS2***	c.467dupT	p.(Leu156Phefs*11)	[298]		
						NNP	*NPHS2*	c.686G>A	p.(Arg229Gln)	[69]		
117	F	1.4	SRNS	In; ND; ND	Not done	LP	***NPHS2***	c.872G>A	p.(Arg291Gln)	[299]		
						LP	*NPHS1*	c.2512C>A	p.(Pro838Thr)	PS	Del; 1.00	NL; NL; NL

* Denotes age at genetic testing where age at disease onset was not available.

Legend:

AA, amino acid; CNS, congenital nephrotic syndrome; FH, family history; FSGS, focal segmental glomerulosclerosis; In, Indian; LP, likely-pathogenic; mat, maternal; N, no; ND, not done/no data; NL, not listed; NNP, non-neutral polymorphism; pat, paternal; PS, present study; SRNS, steroid resistant nephrotic syndrome; W, White; Y, yes

Three of these patients (112, 113 and 114) with early-onset SRNS were heterozygous for previously-reported rare pathogenic variants in *NPHS1*. Patient 112 developed SRNS under the age of 4 years with a heterozygous p.(Asp105Asn) variant previously reported in a Japanese CNS patient where a second variant was not detected [294]. Patient 113 had a heterozygous p.(Arg299Cys) variant. Patient 114, from a Jordanian consanguineous family, who presented with CNS and FSGS on biopsy had a c.1138C>T, p.(Gln380*) nonsense variant but, in common with the other cases, no other likely-pathogenic variants in *NPHS1* or other genes in the panel. Patient 117 had a single heterozygous missense variant in *NPHS2*: c.872G>A, p.(Arg291Gln) previously reported as pathogenic in the homozygous/compound heterozygous state. Patient 117 also had a novel single heterozygous variant in *NPHS1*: c.2512C>A, p.(Pro838Thr) which is not reported in population databases and prediction tools suggest is deleterious.

Two further patients (115 and 116) presenting with classical NS had single previously-reported pathogenic variants in *NPHS2* p.(Arg138Gln) (exon 3) and p.(Leu156Phefs*11) (exon 4) in a compound heterozygous state with the *NPHS2* non-neutral polymorphism p.(Arg229Gln). Tory *et al.* (2014) previously demonstrated that p.(Arg229Gln) is only pathogenic in combination with variants in exons 7 or 8 and, therefore, should not be pathogenic with p.(Arg138Gln) or p.(Leu156Phefs*11) [69]. It is possible that a third intronic or promotor variant in *NPHS2* is contributing to these patients' phenotypes.

4.3.8 Likely pathogenic variants by age of disease onset

The age of disease onset was known with certainty for 164 (64.3%) of 255 patients referred with a clinical presentation of SRNS and Figure 4.4 illustrates the genetic diagnostic rates by age group. In patients with CNS the diagnostic rate was 58.1% (18/31) with LP variants in the following genes: *NPHS1* (12 patients), *LAMB2* (3 patients), *NPHS2* (2 patients) and *WT1* (1 patient). For cases of SRNS with known age of onset over 18 years the rate was 28.6% (6/21) with LP variants in: *INF2* (2 patients), *WT1* (2 patients), *SCARB2* (1 patient) and *TRPC6* (1 patient).

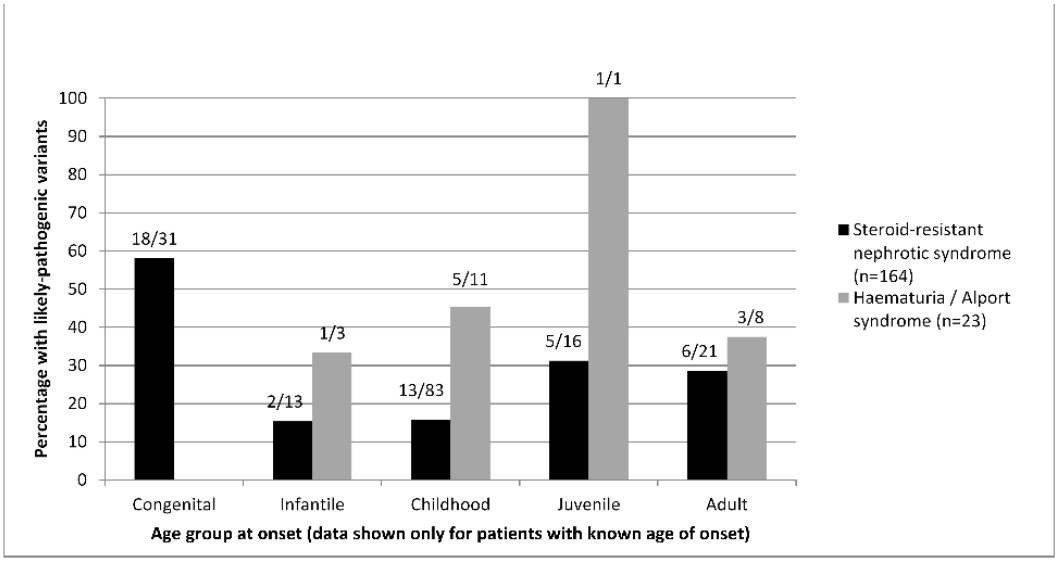

Figure 4.4: Genetic diagnostic rates for patients with known age at disease onset

Figures above each bar indicate: number with genetic disease / total number of patients in each group. None of the 12 patients with steroid-sensitive nephrotic syndrome had likely-pathogenic variants and so are not shown in this figure

4.3.9 Clinical impact of genetic testing

Physicians were asked whether the results of genetic testing would alter immunosuppressive management in patients with SRNS. Responses were obtained in relation to 71 (27.8%) of these patients. The response rate was not high enough to make definitive conclusions, but broadly clinicians reported that results would influence decisions to reduce or stop immunosuppression and one physician indicated that treatment decisions would be made after the gene panel results were known. The diagnostic test result from 67 patients resulted in the subsequent testing of 148 family members including 2 prenatal tests for *LAMB2* and *NPHS2* pathogenic variants. At least 9 family tests are known to have helped inform suitability for donor transplant treatment. Other familial testing has provided diagnoses for relatives and aided in clarifying variant pathogenicity. Other responses confirmed that genetic testing impacts on diagnosis and prognosis after transplant.

4.4 Discussion

Gene panel testing is becoming more and more relevant for screening rare diseases, with greatly increased cost/benefit. The knowledge of the genetic basis of SRNS has expanded considerably over the past 5 years with several national and international cohorts recently published [1, 44, 57] where genetic analysis was undertaken either as part of a research study or on a limited number of genes dependent on the country or institution where the patient was seen. This study reported on large gene panel testing available on a clinical diagnostic basis within the National Health Service in the United Kingdom. Analysis was performed at a single accredited centre, with technological and bioinformatics expertise developed over several years in collaboration with academic research institutes. Although the majority of referrals were received from UK clinicians, 41% of referrals were from outside the UK.

The frequency of likely-pathogenic variants among patients with SRNS was 21.2%. This was marginally lower than 24-34% reported in other studies which included predominantly subjects with childhood-onset disease [44, 57, 59, 282]. The cohort reported here included patients referred for genetic testing by clinicians in routine practice and was, therefore, more heterogeneous than those included in international registries of SRNS. The cohort was also not restricted to patients with childhood-onset disease with 30 patients having known onset in adulthood. As illustrated in Figure 4.4, the genetic diagnostic rate decreased with increasing age of onset from congenital to childhood, similar to that reported in another international cohort [57]. However, there was an increased diagnostic rate in the juvenile and adult subgroups. It is likely that the adults referred by

clinicians for gene panel testing were a filtered population of cases as suggested by the higher frequency of a positive family history of 52.6%.

Twelve patients with SSNS were referred for genetic testing and none had a potentially pathogenic variant. Thus far, no inherited causes for SSNS have been identified [300]. Associations between SSNS and variants in *EMP2*, *KANK1* and *KANK2* have been described in family studies but evaluation in larger cohorts did not identify additional patients with pathogenic variants [108, 112]. *KANK2* was included in the gene panel reported here and no likely pathogenic variants were identified. The updated version of the panel includes *EMP2, KANK1* and *KANK4*. Our current recommendation for clinicians is not to use the NGS gene panel test for patients with persistently steroid-sensitive NS unless there are specific reasons to suspect a genetic aetiology.

In this study, 100% of LP variants identified by NGS meeting diagnostic variant-calling quality parameters have subsequently been confirmed as being present on Sanger sequencing. Sanger confirmation of NGS LP variants is currently in line with best practice ACGS guidance. It also provides a confirmation of sample identity following pooling of samples during the NGS process and establishes a familial test for relatives. As further evidence is collected, confirmatory testing may become redundant.

The use of gene panel testing supersedes stepwise screening protocols [59] and avoids phenotype selection bias, allowing detection of pathogenic variants in genes that would not necessarily be expected from the clinical presentation such as the two adult cases with a *WT1* variant without any manifesting extra-renal features. In addition, variants in secondary genes which may potentially contribute to the phenotype of the patient can be identified by a panel approach.

Gene panel screening identified 32 likely-pathogenic variants without a previous disease association. Absence or rarity in population data is used as evidence to support pathogenicity, however it is acknowledged that some ethnicities in this global cohort were either not known or may currently be insufficiently represented in population databases. Where possible, segregation analysis was performed to provide additional evidence to support pathogenicity, however family members were not always available for testing, reflecting the use of this panel in a clinical setting.

The interpretation of variants of unknown significance in a global cohort is also a challenge of panel testing. As well as segregation analysis, future expansion of population databases will allow improved filtering of population specific variants and functional work may also aid the interpretation of variants.

This study has demonstrated two cases of CNVs present in NS genes, therefore CNV analysis of NGS data should be routinely undertaken as part of the variant analysis pipeline together with confirmation using a second method such as MLPA. It is also apparent that there are a number of clinically typical cases with only a single known *NPHS1* or *NPHS2* pathogenic variant detected, suggesting deep intronic or regulatory variants if they are truly inherited in a recessive pattern. Future whole genome sequencing in these patients may help to elucidate a genetic pathogenesis.

The timing of testing in relation to disease onset and the speed of genetic reporting are important for clinical utility. It is potentially possible to generate results within 1-2 weeks thereby avoiding diagnostic biopsy in some cases. In certain contexts, earlier testing and more rapid turnaround are important because results may have direct consequences for prenatal testing and patient treatment.

Pathogenic variants in *COQ2*, *COQ6*, or *PDSS2*, coding for proteins the coenzyme Q_{10} pathway, may indicate the potential for benefit from treatment with this enzyme [119, 301]. Identification of a causative variant may lead to clinicians stopping or avoiding intensification of immunosuppressive treatment. There has, however, been a report of a patient with NS, diffuse mesangial sclerosis and *PLCE1* variants who responded to treatment with steroids and ciclosporin [60]. Cases of unaffected older children and adults with the same homozygous *PLCE1* variants as their affected relatives suggest a more complex genotype-phenotype interplay and raise the possibility of spontaneous improvement rather than a true response to medication [302, 303]. Some patients with *WT1* variants have responded to steroids and immunosuppression [82]. Certain pathogenic variants, such as in *WT1*, should prompt search for other features of an associated syndrome, such as Frasier syndrome and risk of gonadoblastoma [80].

The presence of a causative variant in SRNS is associated with a lower risk of post-transplantation recurrence of disease, occurring in 25.8% of patients testing negative for genetic disease compared with 4.5% of those with an identified variant in a European cohort [44] and 0% in a published UK cohort [1]. Availability of results supporting a genetic diagnosis may prompt more rapid progression to potentially definitive treatment with transplantation rather than persisting with partially-effective medical therapies. Targeted sequencing of family members resulting from gene panel testing has been used prior to transplantation, particularly in cases with autosomal dominant gene variants.

This study has shown that NGS gene panel testing with bioinformatics analysis for pathological SNVs and CNVs at an early stage after diagnosis of

SRNS or suspected Alport syndrome with results in a clinically relevant timescale has the potential to improve patient stratification and the care pathway.

The studies reported thus far have used baseline clinical characteristics and genetics to examine association with long-term outcomes and response to treatment. Subsequent chapters will discuss the investigation of circulating plasma factors, which may have a potential role as biomarkers or involvement in pathogenesis, and the effect of plasma on podocytes' protein expression.

Chapter 5 Plasma Proteomics and Biomarker Discovery

5.1 Introduction

5.1.1 The Human Plasma Proteome

Developments in technology over the past decade have allowed researchers to identify and quantify not just one or a few proteins within a complex mixture but essentially all the proteins using techniques such as mass spectrometry (MS). The analysis can be applied to cell culture extracts, tissue or body fluids. Blood plasma is one of the most complex human-derived proteomes [304]. It includes not only the typical "true plasma proteins" such as albumin, globulins and hormones, which perform their function in the plasma or by circulating to a target organ. It also contains leakage products from damaged cells and aberrant secretions from diseased tissues which have significant potential as biomarkers.

There is a very large dynamic range in protein concentrations in plasma over 10 orders of magnitude. The 22 most abundant proteins, including albumin, represent 99% of the total plasma protein by mass [305, 306]. On the other hand, the least abundant proteins may have important functions including cytokines such as interleukin-6 with a concentration of 0-5 pg/mL. It is within the latter group that proteins with the potential to act as biomarkers are often found.

The Human Proteome Organisation's (HUPO) Plasma Proteome Project, had identified 15710 protein/peptide sequences by 2005, rising to 27351 in the Proteomics IDEntifications Database (PRIDE) by 2011 [307]. By analysis of the MS peptide data, the PeptideAtlas group of researchers generated a non-

redundant, high-confidence list of 1929 proteins in human plasma in the same year [308]. The same group have combined MS data from multiple proteomic experiments to derive lists of the human kidney proteome (n = 5515 proteins), urine proteome (n = 3175 proteins) and plasma proteome (n = 4098 proteins) with the ability to identify proteins which are shared or unique to each [309].

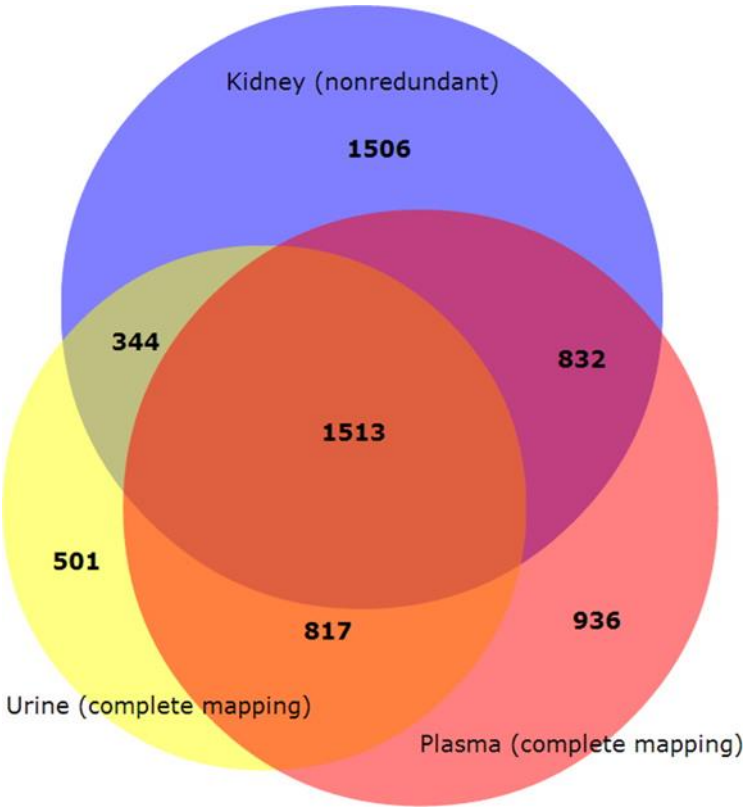

Figure 5.1: Intersection of human kidney, urine and plasma proteomes determined by mass spectrometry

Nonredundant Swiss-Prot protein identifier set for Kidney proteome intersected with the complete mappings for Urine proteome and Plasma proteome. Each Swiss-Prot identifier counted at most once even if multiple distinct splice variants were observed. Reprinted with permission from Farrah *et al*. *J Proteome Res*. 2014, **13**, 60-75.[309] Copyright (2013) American Chemical Society.

Processing of plasma samples prior to MS is important for enabling the greatest utility of data derived from this analysis. The orders of magnitude problem is partially alleviated by depletion of the highly-abundant proteins on the assumption that it is those at lower concentrations that have more potential as biomarkers [310, 311]. Although the depletion step does improve detection and analysis of potential biomarkers, it introduces an additional variable factor which may affect reproducibility [312]. Depletion of albumin and immunoglobulins may lead to loss of proteins bound to them and inevitably there is some variation in effectiveness of depletion from one batch to another. Although the technology for analysis of the whole plasma proteome by MS has been in place for over a decade, the identification and validation of novel biomarkers which have clinical applicability has been limited thus far [305].

5.1.2 Proteomics technology

The typical steps in a proteomic analytical pathway are shown in Figure 5.2.

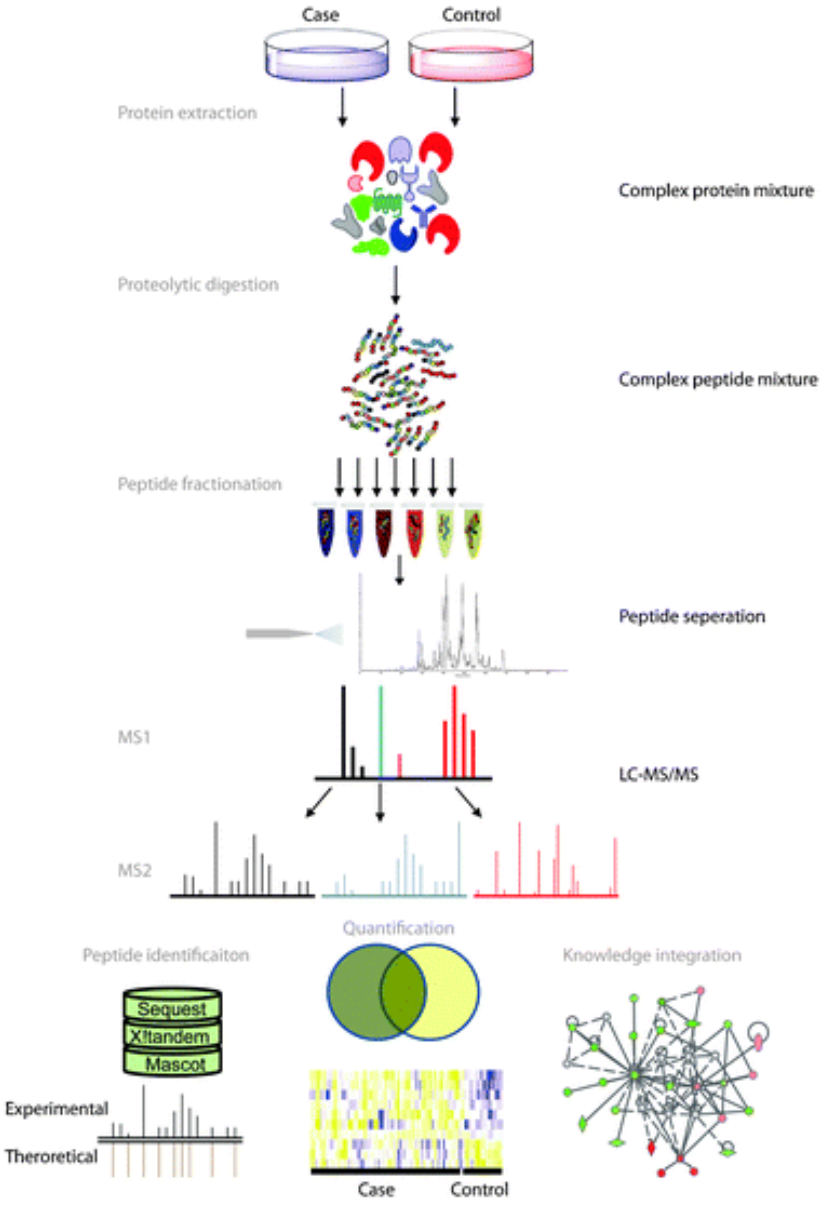

Figure 5.2: Steps in mass spectrometry-based proteomic analysis

Legend: LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry.
Reproduced with permission from The Royal Society of Chemistry, from Angel TE *et al*. *Chem Soc Rev*. 2012;**41**:3912-28.[313]

Typically, an initial method is used to separate the complex mixture of proteins into fractions before analysis by MS, usually two-dimensional gel electrophoresis or liquid chromatography (LC). A wide range of technologies are available but here we will discuss only those used in subsequent parts of the study [314].

First, in the case of complex protein mixtures such as plasma, high abundance proteins including albumin and immunoglobulins are removed to facilitate later analysis of the low concentration, and potentially more interesting, proteins. Equal quantities of protein from each of several samples are prepared. The proteins are digested by trypsin which cleaves on the carboxyl terminal side of arginine and lysine residues except when followed by proline [315]. This typically produces peptides 700 to 1500 Daltons which are the appropriate size for subsequent MS. A key step in multiplex proteomics processing is isobaric labelling. This allows several samples to be mixed and processed together so reducing variability but with the ability to assign the identified proteins to the correct original sample during the data analysis stage. The tandem mass tag (TMT) method allows 10 samples to be processed in parallel using different markers all with same mass which, therefore, do not affect the physical properties in MS but, when fragmented, aid identification (Figure 5.3).

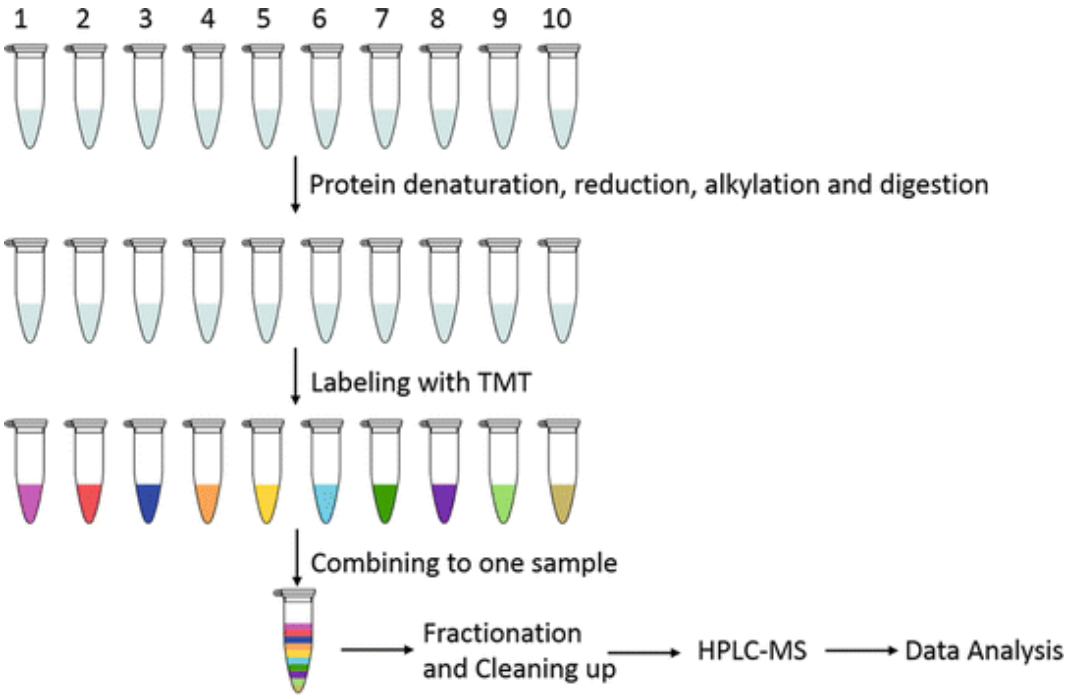

Figure 5.3: Summary of sample processing, isobaric labelling and mass spectrometry for quantitative proteomic analysis

Reprinted by permission from Springer Nature: Relative Protein Quantification Using Tandem Mass Tag Mass Spectrometry by Zhang, L & Elias, JE. [316] Copyright (2017).

After separating the proteins by LC based on mass and charge, the proteins are analysed by MS. At a fundamental level, a mass spectrometer ionises molecules, accelerates them along a curved cylinder and alters their flight path by adjusting the electromagnetic forces. Typically in proteomics, peptides are positively charged and are repelled and accelerated away from the positively-charged cathode. By gradually increasing the AC frequency in the ion trap, peptides of increasing mass to charge (m/z) ratio are emitted and subsequently detected. Within the Orbitrap mass spectrometer, the oscillation of ions within an electromagnetic field is transformed into a mass spectrum which is unique to the m/z ratio of the ion [314]. The experimental spectra are compared, using algorithms such as SEQUEST, against a library of theoretical spectra generated by

in silico trypsin digestion of all human proteins (derived by translation of the genome) [307]. Due to the high stringency of matching criteria, 70% or more of the spectra remain unidentified. Those spectra that are matched enable identification of the peptides and thereby the likely parent proteins. The fragmented TMTs give characteristic peaks on the spectra and thereby allow assignment of peptides and proteins back to the original samples.

5.1.3 Plasma proteomics in SRNS

As discussed in Chapter 1 (Table 1.13), biomarker discovery studies in patients with nephrotic syndrome have been conducted mainly using urine samples and comparing subjects with SSNS and SRNS [317]. In different studies, a variety of analytical techniques were used including MS, SDS-PAGE and ELISA. Two studies have used serum samples to compare FSGS versus MN [245], and SSNS versus SRNS [256] but neither employed MS analysis and therefore the ability to identify novel proteins as potential biomarkers has been limited.

As highlighted in Chapter 1 (Section 1.4.1), several strands of evidence point to the role of circulating factors within plasma as being pathogenic in non-genetic SRNS. Patients with post-transplant recurrence, *ipso facto*, are considered to have circulating factor disease. It can be hypothesised that plasma from such patients at the time of more active disease differs in protein composition, including levels of circulating factors, from plasma at the time of less active disease or remission. Therefore, relative quantification of proteins in plasma using MS and comparison between times of disease relapse and remission has the potential to identify biomarkers associated with these states, some of which may have a role in pathogenesis.

The aim of this study was to compare proteins within plasma at times of disease relapse and remission, and to identify potential biomarkers for future study.

5.2 Methods

5.2.1 Patients

Patients were selected for investigation from the cohort generated by the NephroS study and subjects recruited historically before NephroS was established. Patients were included in the discovery phase if they had a diagnosis of SRNS and if their plasma samples (either derived from blood or plasma exchange) were available at the time of disease relapse and remission. Relapse was defined as uPCR > 200 mg/mmol. Partial remission was defined as uPCR < 200 mg/mmol or plasma albumin > 25. Complete remission was defined as uPCR < 20 mg/mmol. Some patients did not achieve these thresholds but were judged by their treating clinician to have shown relative remission.

In order to reduce variability between patients, all those in the initial discovery group had FSGS on most-recent native kidney biopsy, had progressed to end stage renal failure, received a transplant and suffered disease recurrence in the transplanted kidney. In these cases, samples were derived from plasma exchange. Our group has previously shown that when conditionally-immortalised human podocytes are treated *in vitro* with plasma, vasodilator-stimulated phosphoprotein (VASP) was phosphorylated in response to relapse, but not remission, plasma [188]. Samples from patients in the initial discovery group had previously been tested by collaborators within the group demonstrating consistent differential

phosphorylation of VASP after podocyte treatment with relapse and remission pairs of plasma (D. Henson, personal communication).

Collaboration with Dr Yasuko Kobayashi, also in the Bristol Renal group (now in the Department of Paediatrics, Gunma University Graduate School of Medicine, Gunma, Japan), provided access to plasma proteomics data at times of relapse and remission for 5 patients with SSNS/MCD. These data were used together with results from patients with SRNS in exploratory biomarker analysis as discussed below.

In the validation and further exploratory phases of the study, which involved the use of enzyme-linked immunosorbent assay (ELISA) discussed in Section 5.2.10 below, a more diverse range of patients was included. These comprised patients with:

- SRNS and relapse/remission pairs derived from either plasma exchange or blood plasma
- SRNS and paired plasma samples derived from either plasma exchange or blood plasma but both at times of remission
- SSNS and relapse/remission pairs derived from blood plasma

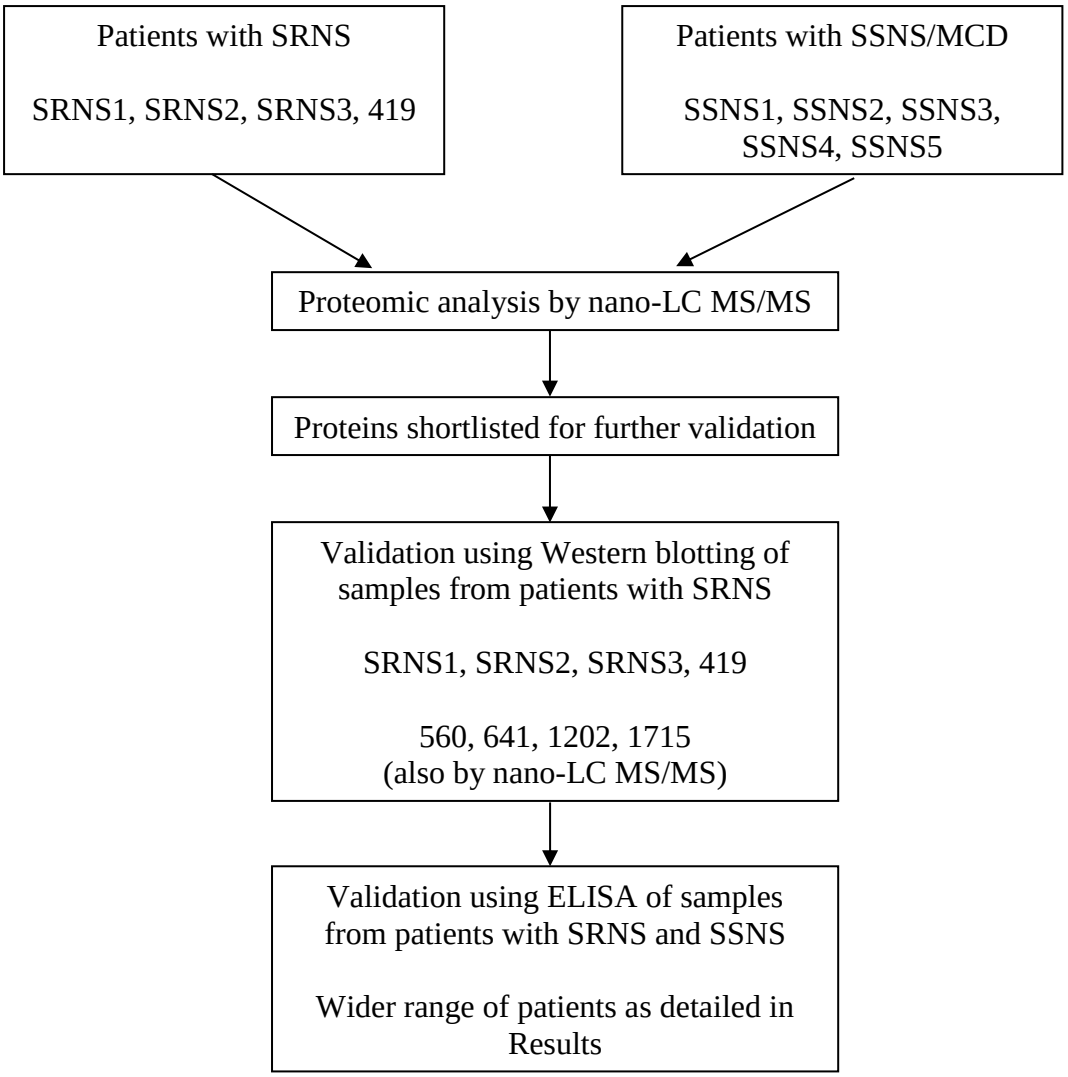

Figure 5.4: Flow diagram showing patients included at different stages of the proteomic discovery and validation process

Legend: ELISA, enzyme-linked immunosorbent assay; MCD, minimal change disease; nano-LC MS/MS, nano-liquid chromatography tandem mass spectrometry; SRNS, steroid-resistant nephrotic syndrome; SSNS, steroid sensitive nephrotic syndrome

5.2.2 Plasma sample processing and storage

Plasma exchange samples were stored at the local centre at 4°C before being couriered in cool bags with ice packs to the central centre. On arrival they were aliquoted for long term storage at -80°C. Blood was collected at local centres into lithium heparin tubes and posted to the central centre at ambient temperature. On arrival they were centrifuged at 3800 rpm at 4°C for 20 minutes. Plasma was removed and aliquoted for long term storage at -80°C. Samples were used where possible after one, but no more than three, freeze-thaw cycles.

5.2.3 Albumin depletion of plasma

Patient plasma samples were retrieved from storage at -80°C and thawed on ice. Albumin & IgG Depletion SpinTrap columns (GE Healthcare Life Sciences, Little Chalfont, UK; #28-9480-20) were prepared and used following the manufacturer's instructions. Briefly, after shaking the columns they were placed in 2 ml microcentrifuge tubes, centrifuged for 30 s at 100 × g and the collected liquid discarded. The columns were equilibrated with binding buffer (15.5 mmol/L Na_2HPO_4, 4.5 mmol/L NaH_2PO_4, 150 mmol/L NaCl, pH 7.4) by adding 400 µL, centrifuging for 30 s at 800 × g and discarding collected liquid. This step was repeated once. The columns were placed in a new 2 mL microcentrifuge tube. 50 µL plasma was mixed with 50 µL binding buffer, applied to each column and incubated for 5 minutes without mixing. Columns were centrifuged for 30 s at 800 × g and the eluate collected. A further 100 µL binding buffer was added to each column and centrifuged for 30 s at 800 × g. This step was repeated once to give a final volume of 300 µL. The total protein concentration was determined using the bicinchoninic acid (BCA) assay following the manufacturer's

instructions (Pierce®, Thermo Fisher Scientific, Boston, MA, USA; #23225) and 100 μg protein sent on ice to the Bristol Proteomics Facility for total proteomic analysis. Remaining albumin-depleted plasma was stored at -80°C for later analysis.

5.2.4 Tissue culture of conditionally-immortalised human podocytes

Conditionally-immortalised human podocytes were derived from normal patient samples using the processes described previously [187]. Frozen stocks of podocytes were thawed from -80°C and transferred to a T75 vented flask before adding standard medium (RMPI 1640 [Sigma-Aldrich, Poole, UK; #R8758] supplemented with 10% heat-inactivated fetal bovine serum (FBS) [Thermo Fisher Scientific; #10500-064] and 1% insulin-transferrin-selenium (ITS) [Thermo Fisher Scientific; #41400-045]). Medium was changed three times per week and cells kept at 33°C in 5% CO_2 until above 80% confluence. Cells in the stock flask were released with 0.5 mL trypsin-EDTA (Sigma-Aldrich; #T4049), suspended in 10 mL standard medium and centrifuged at 1200 rpm for 5 minutes.

The cell pellet was resuspended in 8-10 mL. In the case of T75 flasks, 1 mL was aliquoted to the flasks before adding 9 mL standard medium and returning to 33°C. For 6-well plates, the cells suspension was diluted 1:12.5 with medium and 2 mL aliquoted to each well of the plate. When the cells reached 70-80% confluence they were thermoswitched to 37°C to promote terminal differentiation. Cells were used for experiments at day 10-14 post-thermoswitching.

5.2.5 Treatment of podocytes with albumin-depleted plasma

On the day of treatment, standard medium was removed and replaced with RMPI 1640 lacking FBS and ITS. Podocytes were incubated for 2 hours in serum-free medium (SFM) before treatment started. Albumin-depleted plasma was diluted in serum-free RPMI 1640 to a final concentration of 10%. Paired relapse and remission samples were used. A final volume of 4 ml per T75 flask and 0.5 mL per well of a 6-well plate was used. Treatments and controls were as follows:

1. SFM
2. Albumin binding buffer (ABB, as detailed in Section 5.2.3) 60% : SFM 40%
3. Flufenamic acid (Sigma-Aldrich; #F9005) 200 mmol/L in dimethyl sulfoxide (DMSO, Sigma-Aldrich; # D2650) diluted to 200 µmol/L in SFM
4. Control patient plasma (C1) 10% : ABB 50% : SFM 40%
5. Albumin-depleted (AD) or non-AD relapse patient plasma 10% : ABB 50% : SFM 40%
6. AD or non-AD remission patient plasma 10% : ABB 50% : SFM 40%

Flufenamic acid (FFA) has been shown to activate the cation channel TRPC6 leading to intracellular calcium signalling in culture podocytes [318]. This results in VASP phosphorylation hence FFA treatment acts as a positive control in these experiments.

After applying the treatments as listed above, podocytes were returned to 37°C for 30 minutes.

5.2.6 Protein extraction and preparation

5.2.6.1 Solutions used for protein extraction

The solutions used for protein extraction from podocyte cultures are detailed in Table 5.1.

Table 5.1: Solutions used for protein extraction from podocyte cultures

1 × Phosphate buffered saline (PBS)	135 mmol/L NaCl
	3 mmol/L KCl
	1 mmol/L Na_2HPO_4
	1.75 mmol/L KH_2PO_4
	pH 7.4
1% Triton extraction buffer	150 mmol/L NaCl
	20 mmol/L Tris base
	10% (v/v) Glycerol
	1% (v/v) Triton® X-100
Supplements added to 1% Triton buffer just prior to use	1% (v/v) Protease inhibitor cocktail (Sigma-Aldrich; #P8340)
	1% (v/v) Phosphatase inhibitor cocktail 3 (Sigma-Aldrich; #P0044)
	400 µmol/L Phenylmethane sulfonyl fluoride (PMSF)

5.2.6.2 Protein extraction method

Flasks of treated podocytes were removed from the 37°C incubator and placed immediately on ice. The cells were washed twice with ice-cold PBS and all fluid removed before adding 300 µL per flask or 100 µL per well of 1% Triton extraction buffer with supplements. Cells were scraped from the base of the tissue culture flask and centrifuged at 13200 rpm at 4°C for 5 minutes to remove cellular debris. The supernatant was transferred to fresh Eppendorfs and stored at -80°C.

5.2.7 Quantitative total proteomics

Quantitative total proteomics of albumin- and IgG-depleted plasma was performed at the University of Bristol Proteomics Facility under the direction of Dr Kate Heesom as previously described [319]. Briefly, 10 samples were digested with trypsin overnight, labelled with Tandem Mass Tag (TMT) 10-plex reagents and pooled together. For total proteomics, the pooled samples were fractionated by high-pH reversed-phase chromatography. The fractions were analysed by nano-liquid chromatography tandem mass spectrometry (nano-LC MS/MS) using an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific). Data files were processed and analysed with Proteome Discoverer software (v1.4, Thermo Fisher Scientific) and searched against the Uniprot Human database (134169 sequences) using the SEQUEST algorithm. The search was performed with full tryptic digestion allowing a maximum of one missed cleavage. The false discovery rate (FDR) was set at 5% for all peptide data using the reverse database search option.

5.2.8 Total Proteomics data analysis

Quantitative proteomic data were analysed using Proteome Discoverer software (v4.1, Thermo Scientific) and Excel (v2007, Microsoft). During the processing of mass spectra in Proteome Discoverer, the algorithms sometimes assigned different (protein) accession numbers to peptides from the same or highly related proteins (for example protein fragments or different isoforms). Figure 5.5 shows a snapshot from Proteome Discoverer of one protein group with 6 members.

Accession	Description
C9J8S8	Receptor-type tyrosine-protein phosphatase delta OS=Homo sapiens GN=PTPRD PE=1 SV=1 - [C9J8S8_HUMAN]
Q59H90	Protein tyrosine phosphatase, receptor type, D isoform 4 variant (Fragment) OS=Homo sapiens PE=2 SV=1 - [Q59H90_HUMAN]
Q3KPI9	PTPRD protein OS=Homo sapiens GN=PTPRD PE=1 SV=1 - [Q3KPI9_HUMAN]
F5GWR7	Protein-tyrosine-phosphatase OS=Homo sapiens GN=PTPRD PE=1 SV=1 - [F5GWR7_HUMAN]
P23468	Receptor-type tyrosine-protein phosphatase delta OS=Homo sapiens GN=PTPRD PE=1 SV=2 - [PTPRD_HUMAN]
Q2HXI4	Protein-tyrosine-phosphatase OS=Homo sapiens GN=PTPRD PE=2 SV=1 - [Q2HXI4_HUMAN]

Figure 5.5: Snapshot from Proteome Discoverer illustrating protein grouping

Within one dataset, one protein from the group of related proteins was designated by the software as the master protein and the protein group was labelled using the accession number of this master protein (in this case Q59H90). However, in a second dataset, a different master protein/accession number may be used to label the same protein group, and, therefore, the associated quantitative data. When it was necessary to combine results from different proteomic runs, data were first matched using the accession numbers in Excel. Any rows remaining unmatched were compared with the second dataset using the full protein name and/or the gene name. For any rows still remaining unmatched, the protein was identified manually in Proteome Discoverer and the linked accession numbers within the protein group used to search in the second dataset. The aim was to maximise the number of proteins with complete quantitative data for further analysis.

Relative fold change in protein quantification was calculated in Proteome Discoverer using the Ratio Reporting function as the ratio of quantity in the "relapse" sample to quantity in "remission" for the same patient. Data were analysed in three ways.

5.2.8.1 Geometric mean and t tests

The first method aimed to determine both the magnitude and statistical significance of differences in protein quantity between relapse and remission across all patients. The geometric mean of the relapse/remission fold change was calculated for each protein. A paired-sample two-tailed t-test of the $\log_2$ transformed quantification was used to compare the SRNS patient relapse/control with the SRNS patient remission/control data. In order to visualise both magnitude of fold change and statistical significance, ($-\log_{10}$ t-test probability) was plotted against ($\log_2$ geometric mean fold change) to generate a volcano plot [320]. To account for multiple comparisons, the Bonferroni correction was applied (threshold p = 0.05 / number of proteins). An alternative adjustment for multiple comparisons was also used to control the FDR using the Benjamini-Hochberg (BH) procedure [321]. The FDR is the proportion of "discoveries" (test positives) that are actually false positives. The BH procedure involves sorting all p values from smallest to largest and allocating each a rank, *i*, with the smallest being 1. The Benjamini-Hochberg critical value is calculated as $(i/m) \times Q$, where *i* is the rank, *m* is the total number of tests and *Q* is the chosen FDR. The largest p value for which $p < (i/m) \times Q$ is the cut-off for significance and all p values less than this are also significant.

5.2.8.2 Empirical Bayes moderated t tests

A second method of analysis employed a technique recently described by Kammers *et al*. which used empirical Bayes moderated t tests in contrast to the standard t tests described above [320]. This develops on techniques used for gene expression analysis such as LIMMA (Linear Models for Microarray Data).

Given the small number of samples (4 pairs in this case), proteins showing a large fold change may be declared non-significant using standard statistics due to large sample variance. The empirical Bayes procedure uses the whole dataset to reduce the sample variances towards a pooled estimate giving greater ability to identify statistically significant differences.

The data file was opened in Proteome Discoverer with peptide confidence set to "medium" (FDR 5%). The quantification method was set to ratio reporting for the relevant relapse/remission pairs. The experimental bias was set to "Normalize on Protein Median" with a minimum protein count of 20. This mirrored the normalisation step as described by Kammers *et al* [320]. In the normalisation process, each protein relative abundance was divided by the median of all relative protein abundances within a sample resulting in all samples having a median of one. This compensated for any loading/pipetting errors when setting up samples for MS. In Results filters / Peptide grouping, the option to "Show peptide groups" was unselected. In Edit quantification method / Ratio calculation, the option to "Show the Raw Quan values" was selected. These last two steps allowed the quantification data for all individual peptides to be used in subsequent analysis.

The above steps generated a list of 49144 peptides with raw quantification data for each of the two conditions (relapse and remission) for the 4 patients. These data were exported to Microsoft Excel and then filtered to include only peptides used for protein quantification and only peptides with quantification data available for all 8 samples. This reduced the peptide list to 21680.

The data were analysed using the R statistics programme (R version 3.3.2) [322]. The method used software and code developed by Kammers *et al*. and

freely available at http://www.biostat.jhsph.edu/~kkammers/software/eupa/ [320]. The data were imported from Excel and processed in R with full code given at the end of this chapter (Section 5.5.1).

5.2.8.3 Fold change thresholds

In a third method of analysis, the protein quantification ratios for relapse/remission samples were determined in Proteome Discoverer with 5% FDR and without median normalisation. The data were exported to Excel and fold-change thresholds were initially set at > 2 and < 0.5. Proteins were filtered using these thresholds to maximise consistency across patients with paired relapse/remission samples. Less stringent thresholds of > 1.5 and < 0.67, and > 1.3 and < 0.77 were subsequently used to identify more proteins of potential relevance, reaching the threshold across a greater number of patient pairs. Fold change thresholds alone do not take into account the variability of the data [323] but have been widely used previously in proteomics studies related to nephrotic syndrome and other fields of biomedical research [246, 257].

5.2.9 Western blotting

5.2.9.1 Solutions used for gel electrophoresis and Western blotting

Table 5.2: Solutions used for gel electrophoresis and Western blotting

Sample buffer	60 mmol/L Tris HCl pH 6.8
	10% (v/v) Glycerol
	2% sodium dodecyl sulphate (SDS)
	0.005% bromophenol blue
	2.5% (v/v) 2-mercaptoethanol (added just prior to use)
10% or 15% Resolving gel	10% or 15% (v/v) acrylamide / bisacrylamide
	375 mmol/L Tris pH 8.8
	0.1% (v/v) SDS
	5 mg/mL ammonium persulphate (APS)
	0.05% (v/v) N,N,N',N'-Tetramethyl ethylenediamine (TEMED)
3.75% Stacking gel	3.75% (v/v) acrylamide / bisacrylamide
	125 mmol/L Tris pH 6.8
	5 mg/mL APS
	0.05% (v/v) TEMED
Running buffer	192 mmol/L glycine
	25 mmol/L Tris pH 8.3
	0.1% SDS
Transfer buffer	192 mmol/L glycine
	25 mmol/L Tris pH 8.3
	10% (v/v) Methanol
Tris-buffered saline with Tween (TBS-T)	137 mmol/L NaCl
	20 mmol/L Tris pH 7.6
	0.1% (v/v) Tween-20®
Blocking buffer	5% bovine serum albumin (BSA, Sigma-Aldrich; #A9647) in TBS-T

5.2.9.2 Gel electrophoresis and Western blotting method

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were used to confirm findings from total proteomics and to analyse proteins in

podocyte extracts. Albumin-depleted plasma was thawed on ice. Using the total protein concentration determined previously by the BCA assay, ABB was added to the samples to give a final protein concentration of 0.5 µg/mL in a total volume of 100 µL. These were mixed 4:1 with 5× sample buffer. Samples were heated at 95°C for 5 minutes in order to denature and reduce proteins. 10%-15% SDS-polyacrylamide gels were prepared depending on the size of proteins to be separated. 25 µL (10 µg) protein samples were loaded in each lane of the gel with molecular weight marker (BLUeye Prestained Protein Ladder, Geneflow, Lichfield, UK; #S6-0024,) at each end. For plasma samples, the same total quantity of protein was loaded per lane based on the BCA assay. For podocyte extracts, 25 µL were loaded per lane without prior protein quantification; instead I used an internal loading control (GAPDH). Gels were immersed in running buffer and a direct current applied at 150 V for 60-75 minutes until the bromophenol blue dye front reached the bottom of the gel and there was adequate separation of the protein sizes of interest. Proteins were transferred onto polyvinylidene fluoride (PVDF, Immobilon-P membrane, 0.45 µm, Merck Millipore, Billerica, MA, USA; #IPVH00010) membranes in transfer buffer at 4°C and 250 mA for 60 minutes. Membranes were blocked in blocking buffer for 1 hour at room temperature. Primary antibodies were prepared in 5% BSA / TBS-T. The antibodies used, and dilutions, are shown in Table 5.3. Membranes were incubated overnight on a rocking platform at 4°C with primary antibodies. The following day, antibodies were stored for re-use at 4°C and membranes rinsed twice in TBS-T before 5 × 5-minute washes. Secondary antibodies were prepared at 1:10,000 dilution in TBS-T and applied for 1 hour at room temperature. Membranes were rinsed and washed as above before application of Clarity

Western ECL substrate (BioRad, Hercules, CA, USA; #170-5061) and imaging with chemiluminescence settings on an Amersham Imager 600 (GE Healthcare Life Sciences).

Table 5.3: Antibodies used in Western blotting

Target	MW (kDa)	Product code	Manufacturer	Species	Dilution
pVASP (Ser157)	50	3111	Cell Signaling Technology (CST), Beverley, MA, USA	Rabbit	1:1000
Lumican	50	H00004060-D01P	Abnova, Taipei, Taiwan	Rabbit	1:1000
Lumican	38, 50, 60	ab98067	Abcam, Cambridge, UK	Rabbit	1:1000
Uteroglobin	10	ab40873	Abcam	Rabbit	1:5000
Nek3	58	ab37636	Abcam	Rabbit	1:1000
RANBP3	70	A301-520A-M	Bethyl Laboratories, Montgomery, TX, USA	Rabbit	1:1000
DAG1	97	GTX105038	GeneTex, Irvine, CA, USA	Rabbit	1:1000
EphB4	135	14960	CST	Rabbit	1:1000
GAPDH	38	MAB374	Merck Millipore	Mouse	1:10000
Rabbit IgG, peroxidase conjugate	-	A6667	Sigma-Aldrich	Goat	1:10000
Mouse IgG, peroxidase conjugate	-	A9044	Sigma-Aldrich	Rabbit	1:10000

5.2.9.3 Quantification of Western blotting

Images of membranes were analysed using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA) [324]. Tif images were converted to grey scale (8-bit) and the rectangular selection tool used to place rectangles around each lane. The "Plot lanes" function was used to create profile plots and horizontal lines drawn across the base of each peak to enclose an area and exclude

background. Vertical lines were drawn to bisect the peaks and the area of the same half of each peak quantified using the "Wand" tool.

5.2.10 ELISA

ELISA was used for quantification and validation of proteins identified by total proteomics of plasma as differentially present in relapse and remission. The following kits were used (Table 5.4):

Table 5.4: ELISA kits used for protein quantification

Target	Product code	Manufacturer
Uteroglobin	RD191022200	Biovendor, Brno, Czech Republic
Lumican	E-EL-H0198	Elabscience, Wuhan, China
Lumican	SEB496Hu	Cloud Clone Corp, Katy, TX, USA

The manufacturers' protocol was followed with single wells used for the standard curve and test samples in duplicate for trial kits (24 wells) and all standards and samples in duplicate for 96-well plates.

For uteroglobin ELISA analysis, the manufacturer recommended 4-parameter logistic regression (4PL) fitting for the standard curve. This was implemented using freely available software online at http://www.elisaanalysis.com. The 4PL method produced a curve of best fit for the standards assuming an (approximate) symmetrical S-shape on logarithmic axes with upper and lower detection limits [325, 326]. The regression equation was in the form:

$$y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^b}$$

where y is the measured optical density (OD), x is the uteroglobin concentration and a to d are the four parameters. A separate curve was generated using the standards for each ELISA plate. The 4PL curves were visualised and compared with the polynomial (third, fourth or fifth order) lines of best fit for the standards in Microsoft Excel to confirm that the interpolated concentrations for the test samples appeared reasonable.

Product literature accompanying the uteroglobin ELISA (Biovendor) reported a mean serum concentration of uteroglobin of 6.4 ng/mL (SD 1.9, range 3.7-9.4, n = 18) for healthy men aged 20-39 years and mean 7.3 ng/mL (SD 2.3, range 3.6-11.1, n = 12) for healthy women of the same age. The equivalent data were not available for plasma, however the mean lithium heparin plasma / serum concentration for a different set of subjects was 98.3%. No results were provided for children.

5.2.11 Statistical analysis

Data were analysed using Microsoft Excel 2016 and GraphPad Prism 7. The geometric mean was used to summarise fold change data across multiple patients. Statistical analysis was undertaken on the $\log_2$ transformed values. The paired t test (with two tails) was used to compare protein quantification data at the time of relapse and remission. The Bonferroni correction and Benjamini-Hochberg procedures were used to account for multiple comparisons as described previously. The association between variables was determined using Spearman's rank correlation where bivariate normal distributions could not be assumed. The threshold for rejecting the null hypothesis was set at $p < 0.05$.

5.3 Results

5.3.1 Patient characteristics

Investigations using patient plasma samples during this study started in September 2014. During the course of the project, more patients were recruited to RaDaR and NephroS and additional samples became available for testing. Table 5.5 details the characteristics of patients included at one or more stages of the investigation of plasma proteins as biomarkers in nephrotic syndrome.

Patients C1 and C2 were control subjects, both of whom had PEx for indications other than SRNS. C1 had cryoglobulinaemia, an immune-complex-mediated systemic vasculitis, without any renal involvement. C2 had granulomatosis with polyangiitis (Wegener's granulomatosis), an anti-neutrophil cytoplasmic antibody (ANCA)-associated small vessel vasculitis, with renal involvement [327]. These disease processes may alter the plasma proteome but would not affect the analyses in this study based on comparing relapse and remission plasma samples from the same patient with SRNS.

As discussed below, plasma samples from two sets of four patients were analysed on separate occasions by MS. The first group was SRNS1, SRNS2, SRNS3 and 419. The second group, whose samples were received later in the study, comprised 560, 641, 1202 and 1715. All patients had a diagnosis of SRNS, had progressed to ESRF and been transplanted, and suffered post-transplant recurrence. Among patients who had had genetic testing, none had a genetic cause for disease identified. The timing and laboratory results associated with the plasma samples are detailed in Table 5.6. All samples were derived from plasma exchange except in the case of 1202 where only a blood sample was received at

the time of partial remission. Samples were sent from two patients (REL-1 and REL-2 from 419 and REL from 560) at times when the treating clinical team judged the patients to be in relapse. The plasma was used as "relapse" samples in subsequent experiments. However, when full clinical details and laboratory results became available and were analysed retrospectively, these samples were not at a time when the patients uPCR was greater than 200 mg/mmol.

A wider range of patients was included in subsequent experiments using ELISA to validate proteomics findings. These included 5 patients with SRNS (middle section of Table 5.5 and Table 5.6) and 10 patients with SSNS (lower section of Table 5.5 and Table 5.6)

Table 5.5: Demographic and clinical characteristics of patients included in plasma proteomic studies

Patient	Genetic	Gender	Ethnicity	Familial / Sporadic	Age at onset (years)	Consan-guinity	Resistance to steroids	1st biopsy	CKD stage	Time to ESRF	Transplanted?	Disease recurrence	Follow up (years)
C1	N/A	ND	ND	ND	ND	ND	Cryoglobulin-aemia	ND	ND	ND	ND	ND	ND
C2	N/A	ND	ND	ND	ND	ND	GPA	ND	ND	ND	ND	ND	ND
SRNS 1	ND	M	W	ND	7	ND	SSR	FSGS	5Tx	11	Yes	Yes	15
SRNS 2	ND	F	W	S	26	No	PSR	MCD	5Tx	10.7	Yes	Yes	21
SRNS 3	ND	M	W	ND	7	ND	SSR	MCD	5Tx	9	Yes	Yes	12
419	No	M	W	S	8.0	No	PSR	MCD	5Tx	5.2	Yes	Yes	8.7
560	No	F	I	S	12.1	No	PSR	FSGS	5Tx	1.9	Yes	Yes	5.2
641	No	F	P	S	7.8	ND	PSR	MCD	5Tx	1.8	Yes	Yes	4.2
1202	No	M	W	Fam	13.6	No	SSR	FSGS	5Tx	0.9	Yes	Yes	5.0
1715	No	M	W	S	11.0	No	PSR	Collapsing glomerulopathy	5Tx	0.3	Yes	Yes	2.1
252	ND	F	W	Fam	6.0	No	PSR	FSGS	5Tx	1.6	Yes	Yes	7.5
291	No	F	W	S	9.5	No	PSR	FSGS	5Tx	5.5	Yes	Yes	12.9
1291	No	F	W	S	12.4	No	PSR	FSGS	5Tx	1.3	Yes	Yes	3.0
5618	ND	M	W	ND	6.4	ND	PSR	ND	5Tx	3.7	Yes	Yes	6.7
7030	ND	M	W	ND	1.2	ND	SSR	FSGS	5Tx	16	Yes	Yes	18.5
2375	ND	F	W	S	4	No	FR-SSNS	FSGS	1	N/A	No	N/A	11
2703	ND	F	W	S	5.2	No	FR-SSNS	ND	1	N/A	No	N/A	4.2
2704	ND	F	W + As	S	5.8	No	FR-SSNS	ND	1	N/A	No	N/A	1.4
3496	ND	M	As	S	21.5	No	SSNS	MCD	1	N/A	No	N/A	2.1
3752	ND	M	As	ND	3.3	ND	SDNS	ND	1	N/A	No	N/A	1.0
3889	ND	M	As	S	10.0	No	SDNS	MCD	1	N/A	No	N/A	30.6
6690	ND	M	W	S	4	No	FR-SSNS	MCD	2	N/A	No	N/A	40.4
7025	ND	M	W	S	6.8	No	FR-SSNS	ND	1	N/A	No	N/A	6.7
7944	ND	M	Other	S	33.4	No	SSNS	MCD	1	N/A	No	N/A	9.9
9995	ND	M	As	ND	4.6	ND	SSNS	ND	1	N/A	No	N/A	0.6

The top group of 10 patients (C1-1715) were included in plasma proteomic analyses using mass spectrometry as an initial discovery cohort. The middle group of 5 patients (252-7030) all had SRNS and the bottom group of 10 patients (2375-9995) all had SSNS. Plasma samples from these patients were included in exploratory and validation ELISA studies.
Legend: As, Asian; F, female; Fam, familial; FR, frequently-relapsing; FSGS, focal segmental glomerulosclerosis; GPA, granulomatosis with polyangiitis; I, Indian; M, male; MCD, minimal change disease; N/A, not available; ND, not done / no data; P, Pakistani; PSR, primary steroid resistance; S, sporadic; SDNS, steroid-dependent nephrotic syndrome; SSNS, steroid-sensitive nephrotic syndrome; SSR, secondary steroid resistance; Tx, transplanted; W, white.

Table 5.6: Laboratory results associated with relapse and remission plasma samples used in plasma protein studies

Patient	Diagnosis	Date of transplant	Sample[†]	Sample type	Date of sample	Date of albumin[‡]	Albumin (g/L)	Date of uPCR or dipstick[‡]	uPCR (mg/mmol) or dipstick
SRNS1	SSR	03/01/2011	REL	PEx	10/01/2011	10/01/2011	22	10/01/2011	2190
			P-REM	PEx	07/02/2011	07/02/2011	32	07/02/2011	316
SRNS2	PSR	07/2007	REL	PEx	28/09/2007	24/09/2007	27	24/09/2007	641.7
			P-REM	PEx	16/10/2007	16/10/2007	37	16/10/2007	923.8
SRNS3	SSR	16/04/2005	REL	PEx	14/08/2001	N/A	N/A	N/A	N/A
			REM	PEx	21/11/2005	N/A	N/A	N/A	N/A
419	PSR	21/04/2014	REL-1*	PEx	17/11/2014	17/11/2014	40	17/11/2014	24
			REL-2*	PEx	13/07/2015	13/07/2015	37	13/07/2015	81
			REM	PEx	24/11/2014	24/11/2014	41	24/11/2014	13
560	PSR	23/01/2015	REL*	PEx	11/04/2016	13/04/2016	38	13/04/2016	8
			REM	PEx	20/04/2016	20/04/2016	38	22/04/2016	4 (uACR)
641	PSR	22/11/2015	REL	PEx	22/11/2015	22/11/2015	20	22/11/2015	7797
			P-REM	PEx	21/12/2015	21/12/2015	35	21/12/2015	4348
1202	SSR	29/03/2014	REL	PEx	27/04/2014	26/04/2014	33	26/04/2014	739
			P-REM	Blood	27/05/2014	27/05/2014	32	27/05/2014	159
1715	PSR	23/05/2016	REL	PEx	30/05/2016	30/05/2016	38	30/05/2016	583
			P-REM	PEx	20/06/2016	20/06/2016	51	20/06/2016	42
252	PSR	02/12/2011	REL	PEx	04/12/2011	04/12/2011	26	04/12/2011	1295
			P-REM	PEx	15/12/2011	15/12/2011	32	15/12/2011	529
291	PSR	30/06/2010	REL	PEx	05/07/2010	05/07/2010	14	05/07/2010	500
			P-REM	Blood	05/09/2014	N/A	N/A	05/09/2014	188
1291	PSR	26/05/2016	REL	PEx	29/05/2016	29/05/2016	25	29/05/2016	609
			P-REM	PEx	31/05/2016	31/05/2016	33	31/05/2016	243

5618	PSR	23/03/2017	REL	PEx	30/03/2017	30/03/2017	21	30/03/2017	2915
			REM	PEx	10/04/2017	09/04/2017	34	09/04/2017	9.1
7030	SSR	28/05/2016	REL	PEx	27/06/2016	27/06/2016	41	28/06/2016	360 (uACR)
			P-REM	PEx	03/07/2016	03/07/2016	46	03/07/2016	165 (uACR)
2375	FR-SSNS	N/A	REL	Blood	10/03/2015	15/03/2015	20	15/03/2015	2899
			REM	Blood	21/07/2016	21/07/2016	37	21/07/2016	9.9
2703	FR-SSNS	N/A	REL	Blood	23/11/2015	23/11/2015	25	23/11/2015	453
			P-REM	Blood	23/02/2015	23/02/2015	32	23/02/2015	155
2704	FR-SSNS	N/A	REL	Blood	06/01/2016	06/01/2016	28	05/01/2016	704
			REM	Blood	29/06/2015	29/06/2015	41	N/A	N/A
3496	SSNS	N/A	REL	Blood	17/01/2017	10/01/2017	18	10/01/2017	1988
			REM	Blood	12/04/2016	12/04/2016	44	12/04/2016	0 (dip)
3752	SDNS	N/A	REL	Blood	08/01/2016	08/01/2016	27	08/01/2016	3073
			P-REM	Blood	02/11/2015	27/10/2015	28	14/10/2015	293
3889	SDNS	N/A	REL	Blood	23/08/2016	23/08/2016	14	23/08/2016	1043
			REM	Blood	17/05/2016	17/05/2016	40	17/05/2016	11
6690	FR-SSNS	N/A	REL	Blood	06/12/2016	06/12/2016	24	06/12/2016	565
			REM	Blood	07/06/2016	07/06/2016	35	07/06/2016	11
7025	FR-SSNS	N/A	REL	Blood	03/02/2017	03/02/2017	25	03/02/2017	232
			REM	Blood	24/06/2016	N/A	N/A	25/06/2016	15
7944	SSNS	N/A	REL	Blood	30/08/2016	30/08/2016	31	30/08/2016	891
			P-REM	Blood	16/08/2016	16/08/2016	36	16/08/2016	127
9995	SSNS	N/A	REL*	Blood	19/12/2016	19/12/2016	31	19/12/2016	0 (dip)
			REM	Blood	21/11/2016	21/11/2016	35	21/11/2016	0 (dip)

[†]Due to the need for paired samples for this study, plasma at the time of partial remission had to be used for some patients where no others were available. [*]These samples were taken at a time when the clinical team judged the patient to be in relapse (and were labelled as such). When full data were available and analysed retrospectively the samples were not at relapse defined as uPCR > 200 mg/mmol. [‡]The date of plasma albumin and proteinuria results were as close as possible to the sample date – in most cases this was on the same day.

Table 5.6 Legend: FR-SSNS, frequently-relapsing steroid-sensitive nephrotic syndrome; P-REM, partial remission; PSR, primary steroid resistance; REL, relapse; REM, remission; SSNS, steroid-sensitive nephrotic syndrome; SSR, secondary steroid resistance; uACR, urine albumin:creatinine ratio

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5.3.2 VASP phosphorylation by albumin-depleted plasma samples

Human plasma contains a complex mixture of thousands of proteins. In order to improve detection of low-abundance proteins by MS, it is common practice to deplete samples of albumin and IgG prior to analysis as discussed in Section 5.1.1. Studies performed by the Bristol Renal group have previously demonstrated that treatment of ci-hPod with plasma from patients with SRNS at time of relapse is associated with VASP phosphorylation [188]. In order to assess whether albumin-depletion affected this, podocytes were treated with albumin-depleted and non-albumin-depleted plasma.

Immunoblotting of paired relapse and remission samples both with and without albumin-depletion confirmed that there was no loss of VASP phosphorylation activity with depletion of albumin and IgG (Figure 5.6).

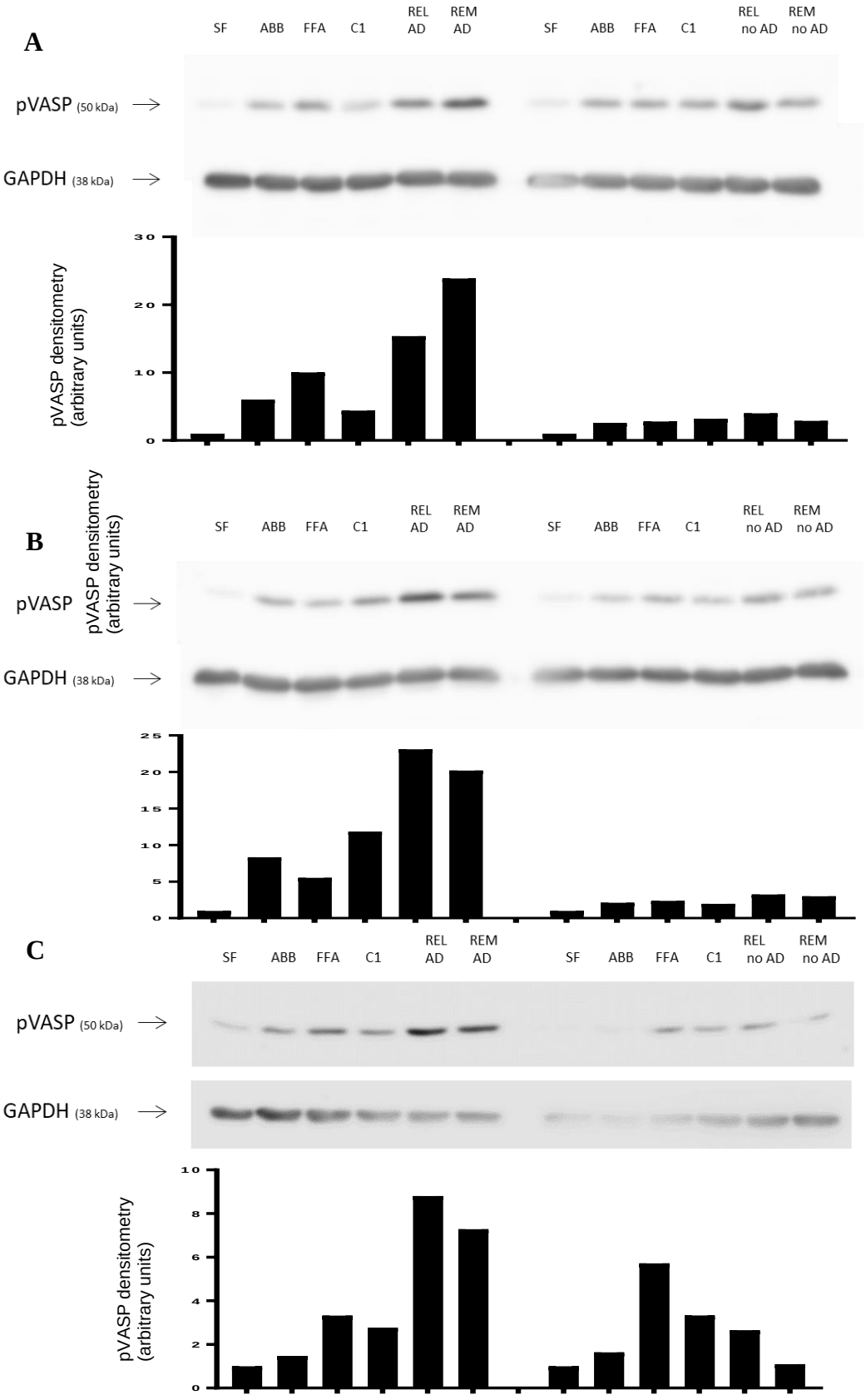


Figure 5.6: Western blots showing VASP phosphorylation in podocytes treated with relapse and remission plasma with or without prior albumin depletion

Podocytes were treated for 30 minutes with plasma at a final concentration of 10%. Phospho-VASP (Ser 157) quantification is shown relative to GAPDH and normalised to serum free control. **A**: Patient 291. **B**: Patient SRNS2. **C**: Patient 419
Legend: SF, serum free media; ABB, albumin binding buffer; FFA, flufenamic acid 200 µmol/L; C1, control patient plasma; REL, relapse plasma; REM, remission plasma; AD, albumin depletion.

For each of the three patients, although the final plasma concentration was the same in all cases (10%) it appeared that VASP phosphorylation was relatively greater with albumin-depleted compared with non-albumin-depleted samples. Albumin-depleted remission plasma from patient 291 (**A** in Figure 5.6) was associated with relatively higher VASP phosphorylation than albumin-depleted relapse plasma which was not expected from other work in the group. Plasma from 291 had shown inconsistency in previous experiments (D. Henson, personal communication and data not shown) and therefore was not selected for further analysis at this stage. Western blotting of albumin-depleted plasma from patients SRNS1 and SRNS3 confirmed that there was no loss of VASP phosphorylation activity (data not shown). Samples from patients SRNS1, SRNS2, SRNS3 and 419, which all showed the expected pattern of VASP phosphorylation after treatment of podocytes and with no loss after albumin depletion, were taken forward for proteomic analysis using LC-MS/MS.

5.3.3 Proteomic analysis of albumin-depleted relapse and remission plasma samples from patients with SRNS

5.3.3.1 Analysis using ordinary t tests

Proteomic analysis of plasma from controls C1 and C2, and paired relapse and remission samples from SRNS1, SRNS2, SRNS3 and 419 identified, in total, 11118 peptides corresponding to 2397 proteins with FDR 5%. Only proteins for which there was complete quantification data for C1 and all patient samples were taken forward for further analysis. This reduced the set to 1926 proteins.

The range of fold change and t-test p values for all proteins are shown in the volcano plot (Figure 5.7).

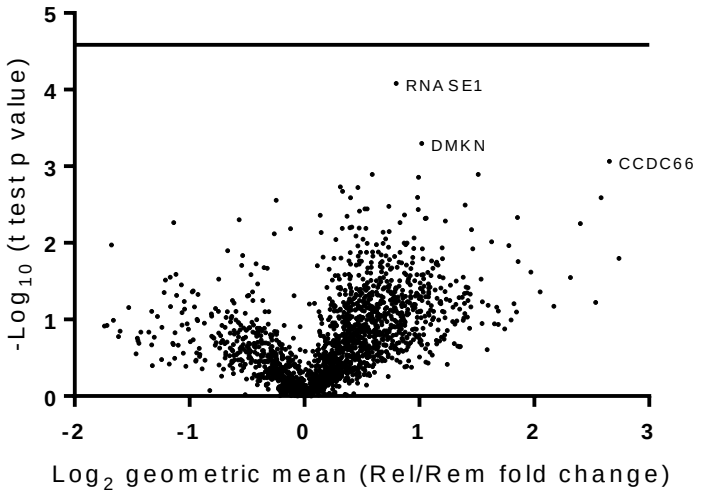


Figure 5.7: Volcano plot of protein quantification data for relapse/remission plasma samples from patients SRNS1, SRNS2, SRNS3 and 419

The horizontal line shows the Bonferroni-corrected significance threshold for 1926 proteins with $\alpha = 0.05$

Among 1926 proteins, 261 (13.5%) achieved nominal significance at the 5% level, however this does not consider the multiple comparisons. The horizontal

line represents the Bonferroni-corrected threshold of 0.05/1926 and it is evident that no proteins reached this level of significance. The three proteins with the most significant fold change were: ribonuclease pancreatic (gene: *RNASE1*, $p = 8.3 \times 10^{-5}$), dermokine (gene: *DMKN*, $p = 5.0 \times 10^{-4}$), and coiled-coil domain-containing protein 66 (gene: *CCDC66*, $p = 8.6 \times 10^{-4}$). No proteins reached the threshold for statistical significance after the Benjamini-Hochberg procedure for multiple comparisons with FDR 10%. There were multiple proteins with Log_2 (geometric mean of relapse/remission fold change) less than -1 or greater than +1 (corresponding to fold change of < 0.5 or > 2 respectively).

As discussed above, the REL-1 sample from patient 419 which was included in the proteomic analysis was taken at a time when the patient was not significantly proteinuric. Patient 419 was therefore excluded, and the same fold change and t test analysis conducted on the remaining 3 patients. The volcano plot representing a total of 1941 proteins is shown in Figure 5.8.

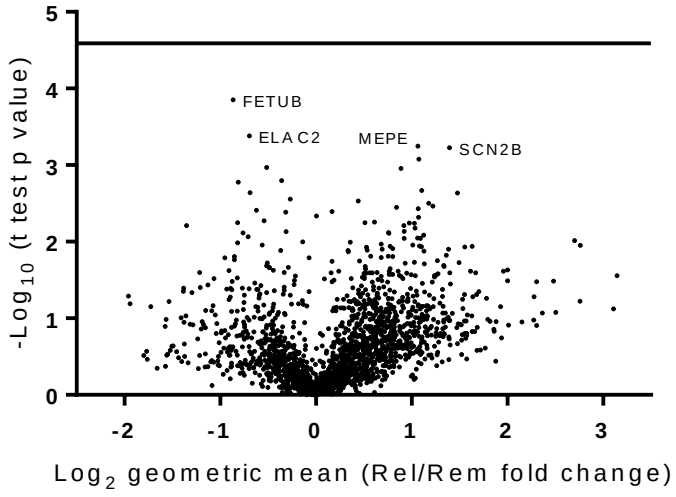


Figure 5.8: Volcano plot of protein quantification data for relapse/remission plasma samples from patients SRNS1, SRNS2 and SRNS3

The horizontal line shows the Bonferroni-corrected significance threshold for 1941 proteins with $\alpha = 0.05$

The three proteins with the most significant fold change were: fetuin B (gene: *FETUB*, $p = 1.4 \times 10^{-4}$), ElaC ribonuclease Z 2 (gene: *ELAC2*, $p = 4.2 \times 10^{-4}$), and matrix extracellular phosphoglycoprotein (gene: *MEPE*, $p = 5.6 \times 10^{-4}$). Again, no proteins reached the Bonferroni-corrected significance threshold or the threshold after the Benjamini-Hochberg procedure.

5.3.3.2 Analysis using empirical Bayes moderated t tests

As discussed above, with small numbers of samples the variance may be large and cause proteins with high fold change to appear non-significant. Moderated t tests were used on median-normalised raw quantification data. Figure 5.9 illustrates the pre-processed normalised protein quantification data for the 8 patient samples. As previously, proteins which could not be quantified due to missing peptide quantification data for some samples were excluded. The analysis used more stringent criteria, namely setting isolation interference < 30% and excluding any proteins only identified by a single peptide. This reduced the total number of proteins in the analysis to 543.

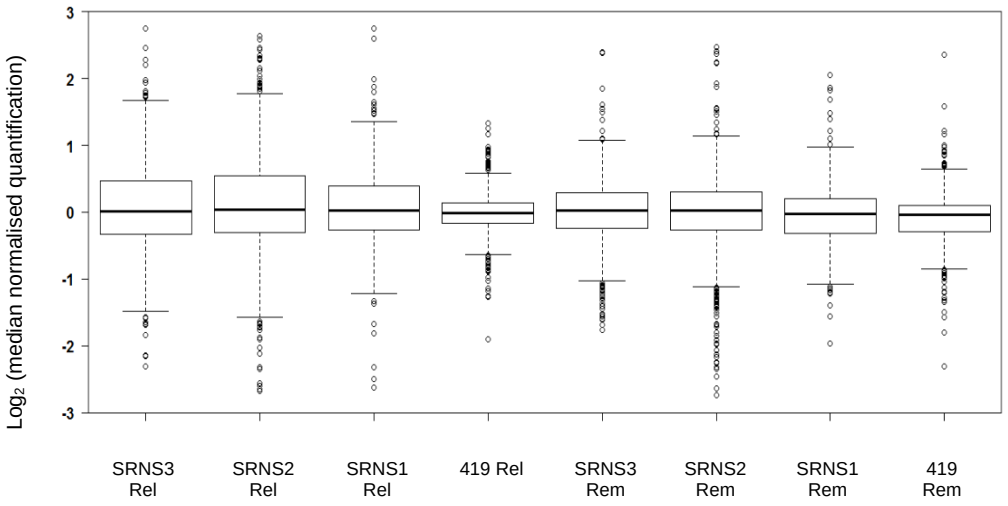


Figure 5.9: Boxplot showing median normalised relative protein abundances in relapse and remission plasma samples from patients SRNS1, SRNS2, SRNS3 and 419

The median-normalised quantification data were analysed in R and volcano plots were generated using ordinary p values and moderated p values (Figure 5.10). Comparison of the plots illustrates that the Bayes moderated t tests generally increased the significance of proteins with large fold change values (and larger variance) and decreased the significance of those with small fold change values (and smaller variance) as was expected [320]. The result was that using moderated statistics, 33 proteins reached nominal significance at a 5% level compared with 27 with ordinary statistics. No proteins reached statistical significance after Bonferroni correction ($p < 9.2 \times 10^{-5}$ corresponding to $-\log_{10}$ p value of 4.04) or the threshold after the Benjamini-Hochberg procedure with FDR 10%.

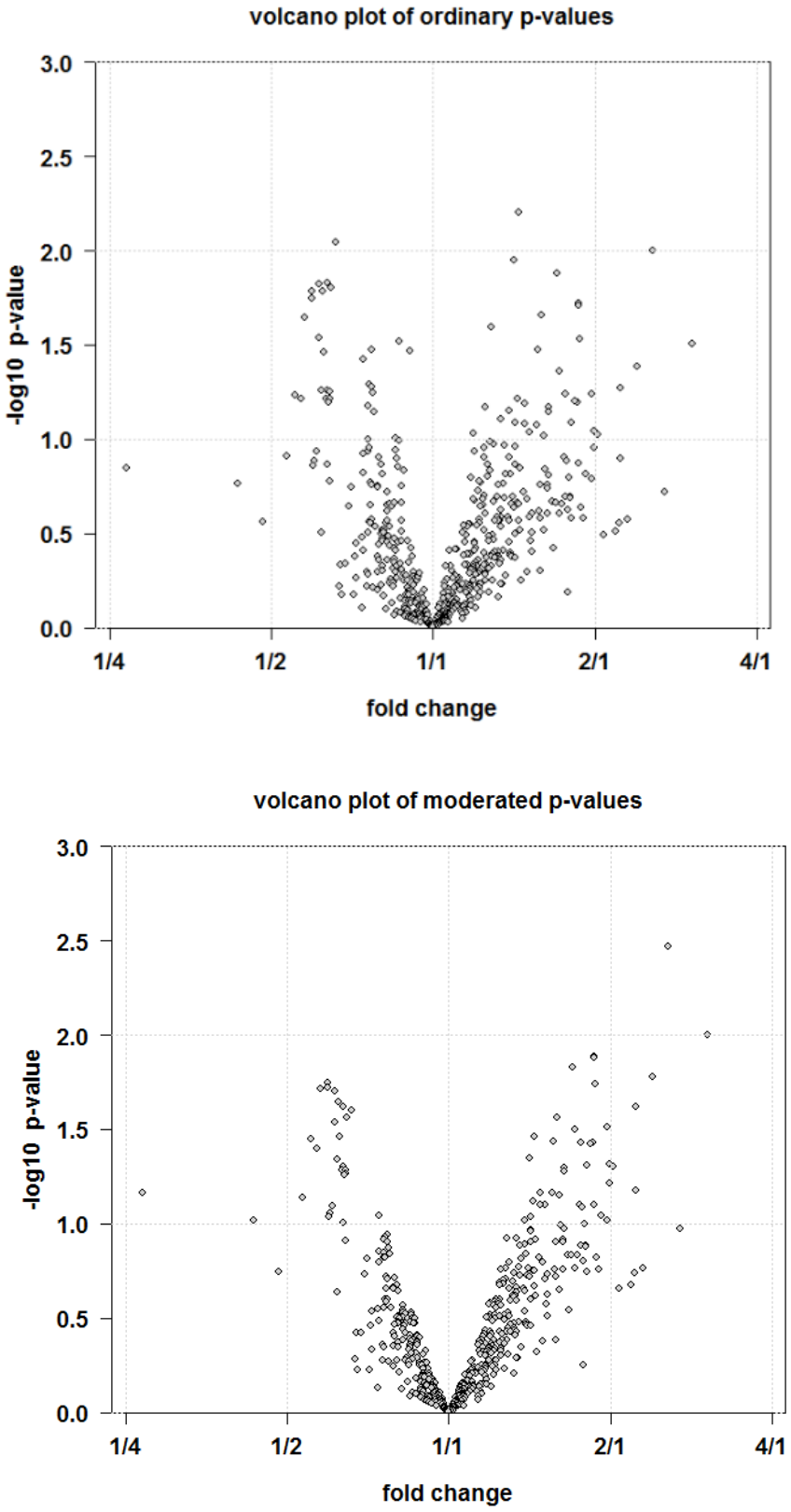


Figure 5.10: Volcano plot of protein quantification data showing ordinary and moderated p values for relapse/remission plasma samples from patients SRNS1, SRNS2, SRNS3 and 419

5.3.3.3 Analysis using fold change thresholds

One of the explanations for the failure of any protein to reach statistical significance was likely to be variability between the small number of samples. In addition, the use of the geometric mean was susceptible to distortion by extreme outliers in one of the patients. As this was an exploratory analysis, it was decided to use fold change thresholds to identify proteins differentially present in relapse and remission. The threshold was initially set to 2 and 0.5, followed by 1.5 and 0.66, and then 1.3 and 0.77 with the aim of identifying proteins consistently changed above or below these values across all four patients. It was considered that fold changes > 1.3 or < 0.77 would be biologically significant. The numbers of proteins reaching the thresholds are shown in Table 5.7 below.

Table 5.7: Number of proteins reaching relapse/remission fold change thresholds for patients SRNS1, SRNS2, SRNS3 and 419

Threshold	Across n patients	Total proteins (n = 1926)	In human plasma proteome database	Proteins reaching threshold using geometric mean
> 2	4	11	2	143
> 1.5	4	37	7	459
> 1.3	4	126	39	745
< 0.5	3*	13	1	42
< 0.66	4	3	0	111
< 0.77	4	14	0	211

*No proteins met the < 0.5-fold change threshold across all 4 patients, therefore the table displays data for proteins meeting the threshold across at least 3 patients

As shown in the table, when a fold change threshold of > 2 was used, 143 proteins reached this based on the geometric mean (that is to the right of the line $\log_2$ geometric mean = 1 in the volcano plots above). However, when the

criterion of consistency across all 4 patients was applied by manual analysis in Excel, this number reduced to 11. The list of proteins reaching threshold in three or four patients was compared with the proteins listed in the human plasma proteome database [328, 329]. Comparatively few of the proteins were found in this database. However, this includes samples only from healthy individuals and proteins were, therefore, not excluded on the basis of not being listed.

A bioinformatic search was conducted using Uniprot [330, 331], STRING [332], NCBI Gene [333] and GeneCards [334] for the proteins reaching the thresholds in 3 or 4 patients as detailed in Table 5.7. The proteins with fold change > 2 or < 0.5 are shown in Table 5.8 giving details of the associated genes and possible protein function. A search for any associations between these proteins and nephrotic syndrome revealed no links. A review of the proteins' functions and subcellular localisation suggested that they were intracellular or membrane bound. Although hypothetically they could be released into the plasma as a result of cell damage it was thought unlikely that they would have a direct function there.

Table 5.8: Proteins identified by mass spectrometry with fold change > 2 or < 0.5 between relapse and remission plasma samples in 4 patients with SRNS

Accession number	Protein name	Associated gene	Possible function	SRNS1 FC	SRNS2 FC	SRNS3 FC	419 FC	Geometric mean FC
Pattern of steroid resistance, first biopsy findings				**SSR, FSGS**	**PSR, MCD**	**SSR, MCD**	**PSR, MCD**	
P78540	Arginase-2, mitochondrial	*ARG2*	Possible role in regulation of extra-urea cycle arginine metabolism	5.32	7.12	18.23	2.87	6.67
F8WB87	Coiled-coil domain-containing protein 66	*CCDC66*	Retinal rod cell development	5.62	5.22	9.41	5.69	6.29
A0A087X128	Protein turtle homolog A	*IGSF9*	Protein binding involved in cell-cell adhesion, regulation of synapse organization	5.18	5.95	10.14	4.12	5.99
F5H176	Nucleosome-remodelling factor subunit BPTF (Fragment)	*BPTF*	Sequence-specific DNA binding, transcription factor binding	3.03	6.44	8.90	4.47	5.28
B3KR25	cDNA FLJ33522 fis, clone BRAMY2006375, highly similar to PIAS-like protein Zimp7	*ZIMZ2*	Zinc ion binding	2.15	6.17	13.79	3.35	4.98
E5RIZ8	Calbindin (Fragment)	*CALB1*	Calcium ion binding	2.50	3.95	13.83	2.16	4.14
K7EN45	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Fragment)	*PIN1*	Peptidyl-prolyl cis-trans isomerase activity	3.89	2.67	5.64	2.92	3.62
A0A087X203	Serine/threonine-protein kinase Nek3	*NEK3*	ATP binding	2.78	3.95	5.87	2.16	3.43
B2RA91	cDNA, FLJ94773, highly similar to Homo sapiens splicing factor, arginine/serine-rich 2, interacting protein (SFRS2IP), mRNA	*SCAF11*	Role in pre-mRNA alternative splicing by regulating spliceosome assembly	3.35	2.32	3.27	2.62	2.86
A0A0G2JMM2	Protein LOC105369239	*LOC105369239*	Multidrug Resistance-Associated Protein 6	2.06	2.60	4.72	2.30	2.76
Q9UG22	GTPase IMAP family member 2	*GIMAP2*	Heterodimer formed by GIMAP2 and GIMAP7 has GTPase activity	2.34	3.60	2.17	2.64	2.64
Q1RLN5	ARHGAP12 protein	*ARHGAP12*	GTPase activator activity; signal transduction	0.43	4.21	0.17	0.19	0.49

H7C4E5	Cytochrome c oxidase copper chaperone (Fragment)	*COX17*	Copper chaperone activity	0.45	0.38	0.35	0.86	0.48
A0A0A6YY96	Iron-responsive element-binding protein 2	*IREB2*	Translation repressor activity	0.29	1.94	0.22	0.38	0.47
Q5T0J7	Testis-expressed sequence 35 protein	*TEX35*	Microtubule cytoskeleton	0.47	0.59	0.35	0.45	0.45
O60503	Adenylate cyclase type 9	*ADCY9*	Catalyses formation of signalling molecule cAMP in response to activation of G protein-coupled receptors	0.41	1.51	0.17	0.19	0.37
Q59GK2	N-deacetylase/N-sulfotransferase (Heparan glucosaminyl) 1 variant (Fragment)	*NDST1*	Heparan sulfate-glucosamine N-sulfotransferase activity	0.33	1.86	0.19	0.15	0.36
B4E205	cDNA FLJ61651, highly similar to Protein transport protein Sec24A	*SEC24A*	Intracellular protein transport	0.04	1.87	0.46	0.45	0.36
A0A024R943	Torsin family 3, member A, isoform CRA_b	*TOR3A*	ATP binding	0.36	1.43	0.10	0.23	0.33
B2RCH7	cDNA, FLJ96082, highly similar to Homo sapiens cervical cancer 1 protooncogene (HCCR1), mRNA	N/A	N/A	0.35	0.21	0.24	0.56	0.31
Q5STZ8	ATP-binding cassette sub-family F member 1 (Fragment)	*ABCF1*	Member of superfamily of ATP-binding cassette (ABC) transporters, lacks transmembrane domains characteristic of most ABC transporters	0.39	1.09	0.09	0.21	0.30
Q59HA5	Cyclin G-associated kinase variant (Fragment)	*GAK*	Protein kinase activity, involved in the uncoating of clathrin-coated vesicles by Hsc70 in non-neuronal cells	1.06	0.26	0.13	0.10	0.24
E5RGB1	F-BAR and double SH3 domains protein 1	*FCHSD1*	Neuromuscular synaptic transmission	N/A	0.08	0.27	0.43	0.21
A0A087WW74	SAM and SH3 domain-containing protein 1	*SASH1*	Scaffold protein involved in the TLR4 signalling pathway	N/A	0.08	0.32	0.35	0.21

Legend: FC, fold change; FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease; N/A, not available; PSR, primary steroid resistance; SSR, secondary steroid resistance

5.3.4 Proteomic analysis of albumin-depleted relapse and remission plasma samples from patients with SRNS and SSNS

Patients SRNS1, SRNS2, SRNS3 and 419 were known to have had recurrence of proteinuria post-transplant suggesting involvement of the putative circulating factor(s) in disease pathogenesis. As discussed in the Chapter 1, MCD is also considered a circulating factor disease[146]. A collaboration with Dr Yasuko Kobayashi, formerly in the Bristol Renal group, provided quantitative proteomic data for relapse and remission plasma samples from 5 patients with SSNS/MCD. All were Japanese with childhood-onset of non-familial SSNS. Three of the five had had biopsies confirming MCD. Clinical and laboratory data relating to the patients are shown in Table 5.9.

Table 5.9: Clinical and laboratory data associated with relapse and remission plasma samples from patients with SSNS

Patient	Gender	Age at onset (years)	Biopsy	Sample	Albumin (g/L)	uPCR (mg/mmol)
SSNS1	M	12.7	ND	REL	41	102
				REM	46	7
SSNS2	M	10.5	MCD	REL	35	1198
				REM	31	0
SSNS3	M	10.5	MCD	REL*	26	10
				REM	46	3
SSNS4	M	1.3	ND	REL	36	283
				REM	35	2
SSNS5	F	3.2	MCD	REL	37	647
				REM	34	11

All plasma samples were from blood. Laboratory results were from the same day as sampling. *This sample was taken at the end of a relapse when proteinuria was improving.
Legend: F, female; M, male; MCD, minimal change disease; ND, not done; REL, relapse; REM, remission uPCR, urine protein:creatinine ratio

It was hypothesised that analysis of the plasma proteome at time of relapse and remission in the four patients with SRNS likely caused by circulating factor disease and the five patients with SSNS may identify a small number of proteins with potential as biomarkers for further investigation. The paired relapse/remission fold-change results from the two datasets were combined in a single spreadsheet using the processes described in Section 5.2.8 to maximise matching of proteins. This resulted in a list of 890 proteins for which there was paired relapse/remission quantitative data for all nine patients.

There were no proteins identified meeting thresholds of (> 2 or < 0.5) or of (> 1.5 or < 0.67) across all nine patients. A compromise needed to be achieved between using a higher fold-change threshold and maximising consistency across the patients. For example, using a fold-change threshold of 2 identified proteins reaching this level in a maximum of four patients and a threshold of 1.5 in a maximum of seven. A fold-change threshold of > 1.3 highlighted 14 proteins which were consistent across seven, eight or all nine patients. These 14 proteins were ranked by geometric mean fold change and, following review of biological information in online databases [331, 333, 334], six of the top seven were chosen for further investigation. These proteins are shown in Table 5.10.

Table 5.10: Proteins consistently increased in relapse versus remission across 4 patients with SRNS and 5 patients with SSNS/MCD

Patient	SSNS1	SSNS2	SSNS3	SSNS4	SSNS5	SRNS1	SRNS2	SRNS3	419
Dystroglycan 1	1.85	0.66	2.10	1.08	1.48	2.34	1.94	3.54	1.32
Receptor protein-tyrosine kinase Ephrin B4	1.69	0.57	1.52	1.59	1.96	2.89	1.41	5.74	1.65
Lumican	1.68	1.51	1.56	1.68	1.62	1.71	1.36	1.81	1.42
Serine/threonine-protein kinase Nek3	1.39	0.94	1.48	1.85	1.40	2.78	3.95	5.87	2.16
Ran-binding protein 3	1.86	0.70	1.89	1.33	0.77	1.68	1.90	3.44	1.96
Uteroglobin	1.35	0.49	1.37	1.10	1.48	2.19	2.03	5.41	1.99

Red highlights fold change > 1.3 and blue < 0.77.

5.3.5 Biological characterisation of selected proteins

The size and known functions of the selected proteins are detailed in Table 5.11.

Table 5.11: Biological characteristics of proteins selected for further investigation

Protein name	Gene	MW (kDa)	Function
Dystroglycan 1	*DAG1*	97	Involved in a number of processes including laminin and basement membrane assembly, sarcolemmal stability, cell survival, peripheral nerve myelination, nodal structure, cell migration, and epithelial polarization
Receptor protein-tyrosine kinase Ephrin B4	*EPHB4*	108	Contact-dependent bidirectional signalling into neighbouring cells; role in heart morphogenesis and angiogenesis through regulation of cell adhesion and cell migration
Lumican	*LUM*	38	Member of the small leucine-rich proteoglycan (SLRP) family; keratan sulfate proteoglycan distributed in interstitial collagenous matrices throughout the body
Serine/threonine-protein kinase Nek3	*NEK3*	58	Member of the NimA (never in mitosis A) family of serine/threonine protein kinases; activated by prolactin stimulation, leading to phosphorylation of VAV2 guanine nucleotide exchange factor, paxillin, and activation of the RAC1 GTPase; regulates microtubule acetylation in neurons
Ran-binding protein 3	*RANBP3*	60	Role in nuclear export as part of a heteromeric complex; cytoskeletal signalling and MAP kinase signalling; negative regulator of TGF-beta signalling through interaction with the R-SMAD proteins, SMAD2 and SMAD3, and mediating their nuclear export
Uteroglobin	*SCGB1A1*	10	Member of the secretoglobin family of small secreted proteins; functions including anti-inflammation, inhibition of phospholipase A2 and the sequestering of hydrophobic ligands.

Data extracted from UniProt and GeneCards. Legend: MW, molecular weight

All proteins had previously been identified in human plasma by MS and were recorded in the Plasma Proteome Database. A search of PubMed was conducted for any associations between the kidneys and/or nephrotic syndrome and the selected proteins.

Dystroglycan has been identified in isolated human glomeruli by immunoblotting and localised to the basal membrane of podocyte foot processes by immunoelectron microscopy [335]. Using kidney biopsy specimens, the study suggested that α- and β-dystroglycan chains were significantly reduced in MCD but not in FSGS. The relative intensity of staining was restored to normal levels after steroid treatment in MCD. More recent studies have indicated that

dystroglycan is expressed by podocytes and has an important, although not critical, role in adhesion [336].

EphB4 is a receptor tyrosine kinase localised to the venous endothelium with a role in angiogenesis. Together with its ligand EphrinB2, it has been identified in human kidney sections and found to be more highly expressed in urogenital tract tumours [337]. EphB4 has been shown to be expressed in healthy podocytes in rats and appeared to be upregulated in a model of nephritis [338]. Inhibition of EphB4 phosphorylation was associated with podocyte damage and increased albuminuria.

Lumican is one of a family of small leucine-rich proteoglycans which also includes decorin, biglycan and fibromodulin. They generally have glycosaminoglycan (GAG) side chains and have a structural role in the extra-cellular matrix [339]. A study of healthy human kidney tissue localised lumican to the glomerular capillary endothelium, particularly in the endothelial cell coat attached to the glycocalyx [340]. Glomerular deposition of lumican has been seen in areas of fibrosis in diabetic nephropathy. Human plasma proteomic studies have previously suggested lumican as a potential biomarker in pancreatic cancer [341] and acute aortic dissection [342, 343].

Nek3 is a member of a family of serine/threonine protein kinases which have a role in microtubule dynamics in cilia and at mitosis [344]. One study has suggested it may function in regulation of cytoskeletal dynamics in neurones. Although *NEK3* gene expression has been reported in microarray studies of normal and diseased kidneys in the Renal Gene Expression Database [345] no specific research has examined the function of the protein in these organs.

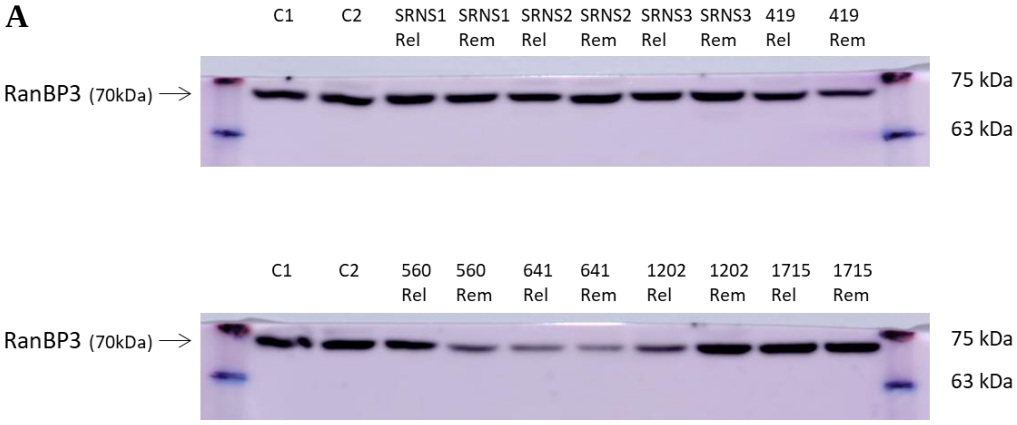
RAN binding protein 3 has a role in nuclear export as a cofactor in a heteromeric complex. In lung cell culture studies it has been shown to be activated by phosphorylation in influenza infection and may have a role in viral ribonucleoprotein export [346]. Another report has suggested a regulatory function in nuclear export in melanoma [347] but no studies have examined a role in the kidneys.

Uteroglobin (also known as Secretoglobin family 1A member 1, Club cell protein 16 or Clara cell 10kDa protein) is a steroid-inducible, secretory protein with immunomodulatory and anti-inflammatory properties acting by blocking soluble phospholipase A2 [348, 349]. It was originally discovered in rabbit uterus but has widespread expression in humans in mucosal epithelia of organs with connections to the external environment as well as being detected in blood, urine and other body fluids. It appears to be involved in development of allergic and inflammatory airways diseases. One study, which measured blood plasma levels using ELISA in patients with pulmonary contusion, has proposed uteroglobin as a biomarker of the severity of injury [350]. An MS-based biomarker discovery study using urine from patients with diabetic nephropathy (n = 6) identified uteroglobin in samples from those with low GFR but not in those with high GFR or healthy controls [351]. Other studies have found higher levels of urinary uteroglobin in patients with proximal tubular dysfunction in CKD and following renal transplant compared with controls [352, 353]. Recent work has suggested that a uteroglobin gene polymorphism (G38A) may be a risk factor for development of idiopathic NS [354]. The AA genotype was associated with a significantly higher chance of both SSNS and SRNS compared with controls.

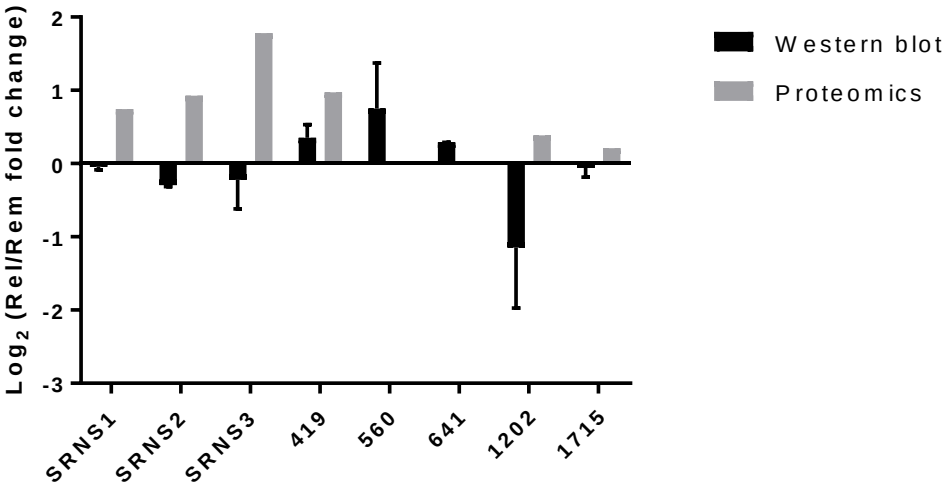
5.3.6 Validation of mass spectrometry proteomics findings by Western blotting

Western blotting was used to validate findings from proteomics focusing initially on the six proteins discussed above in plasma from patients SRNS1, SRNS2, SRNS3 and 419. The same albumin-depleted plasma samples were tested as were used for proteomics. Equal amounts of total protein in the same volume were loaded per lane of the gel. Albumin-depleted plasma relapse/remission pairs from a second cohort of SNRS patients (560, 641, 1202, 1715) were tested in parallel, although the MS plasma proteomic data for them had not yet been analysed at that stage. Figure 5.11 to Figure 5.13 show blots and quantification data for RANBP3, Lumican and NEK3 respectively.

A



B



C

Patient	SRNS1	SRNS2	SRNS3	419	560	641	1202	1715
Western blotting Rel/Rem fold change (geometric mean)	0.97	0.82	0.86	1.28	1.68	1.22	0.45	0.96
Proteomics Rel/Rem fold change	1.68	1.90	3.44	1.96	N/A	N/A	1.31	1.16

Figure 5.11: Western blotting and proteomic quantification of RANBP3 in paired relapse/remission plasma samples

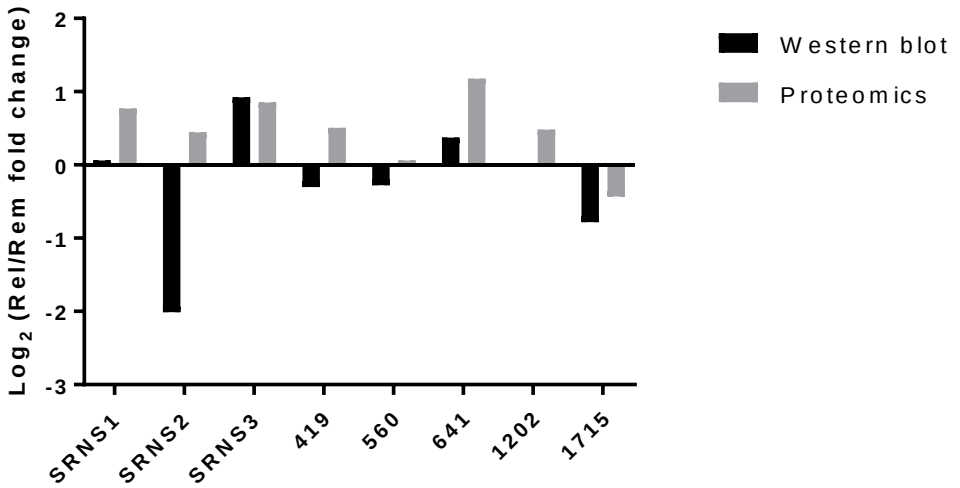
A: Western blot of control (C1, C2) and paired relapse and remission plasma samples. **B**: Mean (SD) of $\log_2$ transformed relapse/remission fold change quantification from densitometry of blots (n = 2) and MS proteomics. **C**: Relapse/remission fold change ratios determined from Western blotting and MS (no MS quantification data were available for RANBP3 in plasma from 560 and 641).

In the case of RANBP3 (Figure 5.11), MS proteomics suggested increased levels in relapse versus remission in the first four patients analysed. When data were later available for the second set, although higher in relapse the fold change was less marked. In two patients' samples (560 and 641), MS was unable to detect RANBP3. In the other cases it was identified by a single peptide.

Western blotting detected a single clear band at the expected molecular weight of 70 kDa (according to antibody product literature, Bethyl Laboratories). No consistent difference between relapse and remission samples was evident either by visualising the bands or by densitometry.

A

B



C

Patient	SRNS1	SRNS2	SRNS3	419	560	641	1202	1715
Western blotting Rel/Rem fold change	1.04	0.25	1.90	0.81	0.83	1.29	N/A	0.58
Proteomics Rel/Rem fold change	1.71	1.36	1.80	1.42	0.99	2.26	1.40	0.74

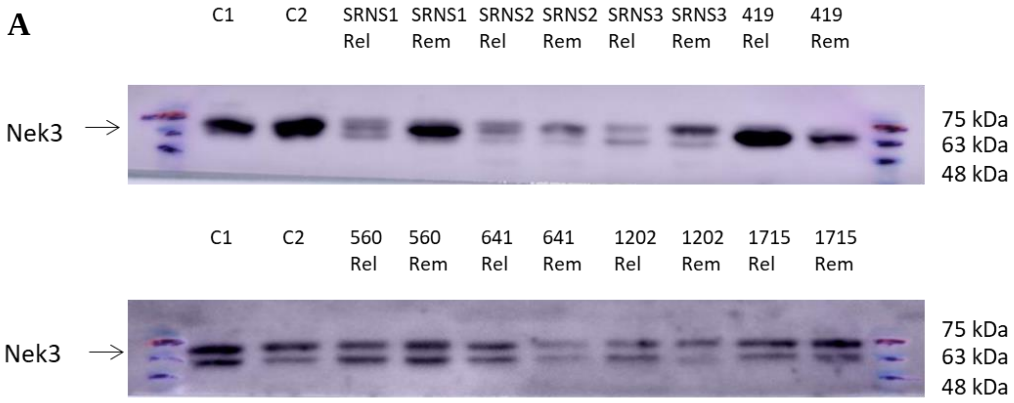
Figure 5.12: Western blotting and proteomic quantification of Lumican in paired relapse/remission plasma samples

A: Western blot of control (C1, C2) and paired relapse and remission plasma samples. **B**: Log_2 transformed relapse/remission fold change quantification from densitometry of blots (n = 1) and MS proteomics. **C**: Relapse/remission fold change ratios determined from Western blotting and MS (densitometry ratio not calculable for 1202).

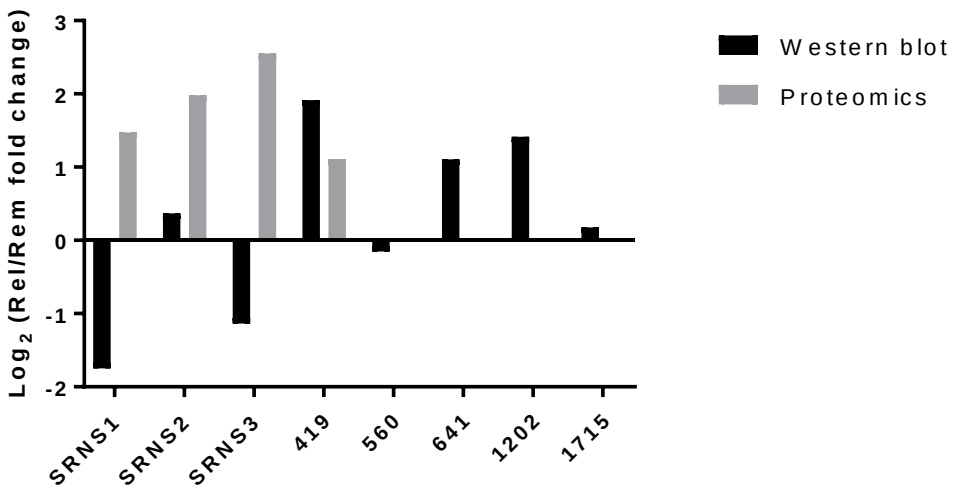
Figure 5.12 shows the results for lumican. MS quantification showed that for two patients in the second set (641 and 1202) the relapse/remission ratio was > 1.3-fold. However, for the other two it was no different or lower in relapse. Western blotting initially followed by detection using an anti-lumican antibody from Abnova (#H00004060-D01P) identified multiple bands at approximately 45, 60, 65 and 75 kDa (data not shown). These bands were persistent in repeat experiments despite changing the blocking agent from 5% BSA to 5% dry milk and additional washes. Figure 5.12A shows a single band at approximately 70kDa identified with a different anti-lumican antibody (Abcam, #ab98067). The predicted molecular weight of lumican based on amino acid sequence was 38 kDa Product literature for the human anti-lumican antibody has reported detection of bands at 50 and 60kDa (Abcam, #ab98067) and 60-85kDa (R&D Systems, #AF2846). These are likely to represent glycosylated forms which have previously been reported [355]. Western blotting showed inconsistency in the relapse/remission ratio for lumican and did not corroborate the MS findings. Due to technical difficulties, ELISA was used as an alternative method for quantifying lumican as discussed below in Section 5.3.8.

Quantification data for Nek3 are shown in Figure 5.13. It was not detected by MS in any of the samples from the second set of patients and was identified by a single peptide in the first set. No, or inconsistent, bands were visible on repeated Western blot experiments and Figure 5.13A shows one result where outcomes were interpretable. The findings did not corroborate the MS data.

A



B



C

Patient	SRNS1	SRNS2	SRNS3	419	560	641	1202	1715
Western blotting Rel/Rem fold change	0.30	1.29	0.46	3.77	0.90	2.15	2.67	1.13
Proteomics Rel/Rem fold change	2.78	3.95	5.87	2.16	N/A	N/A	N/A	N/A

Figure 5.13: Western blotting and proteomic quantification of Nek3 in paired relapse/remission plasma samples

A: Western blot of control (C1, C2) and paired relapse and remission plasma samples. **B**: Log_2 transformed relapse/remission fold change quantification from densitometry of blots (n = 1) and MS proteomics. **C**: Relapse/remission fold change ratios determined from Western blotting and MS (no MS quantification data were available for Nek3 in plasma from 560, 641, 1202 or 1715).

As was the case for Nek3, EphB4 was not detected by MS in any of the samples from the second set of patients. Western blotting on several occasions detected multiple non-specific bands, none of which could be confidently identified as EphB4 with a predicted molecular weight of 135 kDa (Figure 5.14).

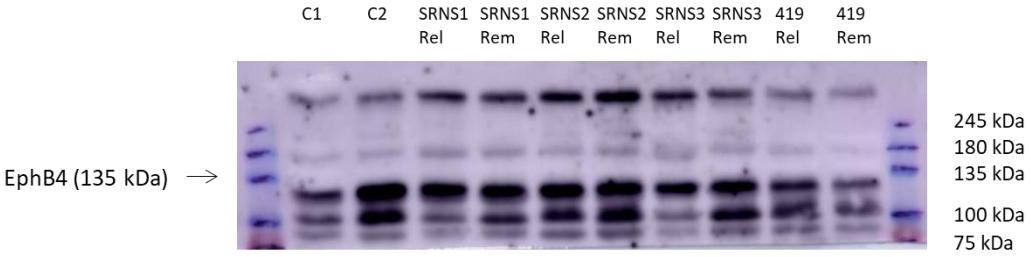


Figure 5.14: Western blotting for EphB4 in paired relapse/remission plasma samples

Western blot of control (C1, C2) and paired relapse and remission plasma samples. The expected position for the EphB4 band was 135 kDa.

Dystroglycan 1 was increased in relapse versus remission plasma > 1.5-fold in patients 560 and 1202 but decreased < 0.77 in the other two. Western blotting did not provide interpretable data.

The relapse/remission ratio for uteroglobin was > 1.3 for patients 560, 641 and 1202 and 0.91 for 1715. Considering all patients together, based on MS quantification, uteroglobin was increased > 1.3-fold in relapse in 7 (88%) of 8 patients with SRNS and 3 (60%) of 5 patients with SSNS. Given the small size of uteroglobin (10 kDa), plasma samples were run on 15% polyacrylamide gel. On several replicates a prominent band was seen at approximately 25 kDa (Figure 5.15) which was still present when blocking with 5% dry milk in place of 5% BSA but absent from lanes treated in the same way but lacking primary antibody in the overnight incubation solution. The band at 25 kDa probably represents non-specific binding to IgG light chains residual in the plasma samples.

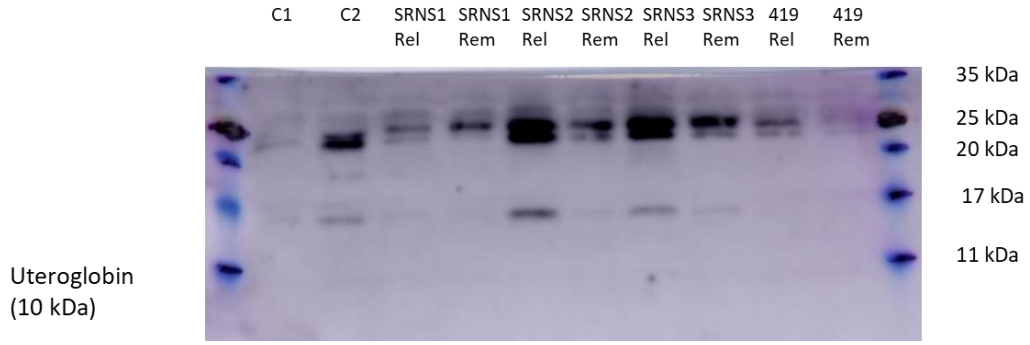


Figure 5.15: Western blotting for Uteroglobin in paired relapse/remission plasma samples

Western blot of control (C1, C2) and paired relapse and remission plasma samples. The expected position for the uteroglobin band was 10 kDa.

Given the consistency noted in the relapse/remission fold change for uteroglobin based on MS proteomics and published literature suggesting potential as a biomarker in other conditions, it was decided to use ELISA as an alternative to Western blotting for uteroglobin quantification.

5.3.7 Validation of mass spectrometry proteomics findings for uteroglobin by ELISA

A Uteroglobin ELISA was identified which had EU approval for *in vitro* clinical diagnostic use (Biovendor, #RD191022200) therefore suggesting a high level of quality control and with prior data from testing samples from healthy subjects. It was first trialled with four paired patient samples. Albumin-depleted plasma samples from patients SRNS1, SRNS2 and SRNS3 were included for consistency with the MS proteomics and Western blotting experiments above. In standard clinical practice, the ELISA does not require the additional steps of albumin- or IgG-depletion and has been used with unmodified plasma samples. Therefore,

non-albumin-depleted plasma from SRNS3 was included for comparison in the initial trial with a view to higher-throughput and larger-scale patient testing.

The standard curve for known concentrations of uteroglobin 0-50 ng/mL determined by the 4PL method (Figure 5.16) and polynomial line of best fit (Figure 5.17) are shown below. Both curves showed a good fit to the measured standards and similar calculated values for the concentration of uteroglobin in the plasma samples. The 4PL method was used for subsequent uteroglobin ELISA analysis.

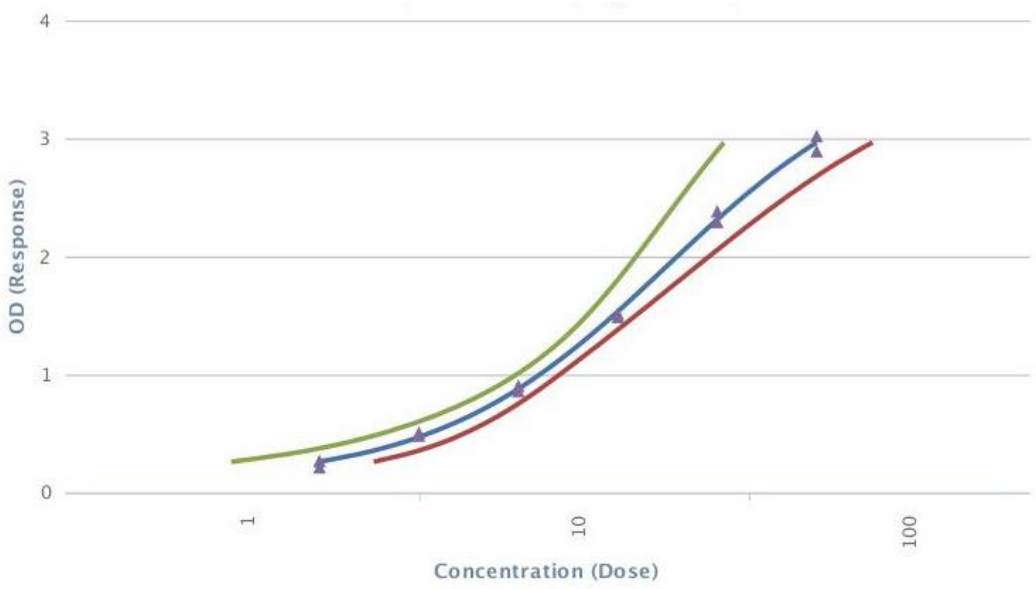


Figure 5.16: ELISA standard curve of optical density against uteroglobin concentration with curve fitting using the 4-parameter logistic regression method

Absorbance was measured at 450 nm with the reference wavelength set to 630 nm. The concentration (ng/mL) is shown on a logarithmic scale. The measured standards (in duplicate) are shown in grey with the line of best fit (blue). The 95% upper (green) and lower (red) confidence bounds are also shown. The coefficient of determination (R^2) = 0.998. The curves were generated using www.elisanalysis.com.

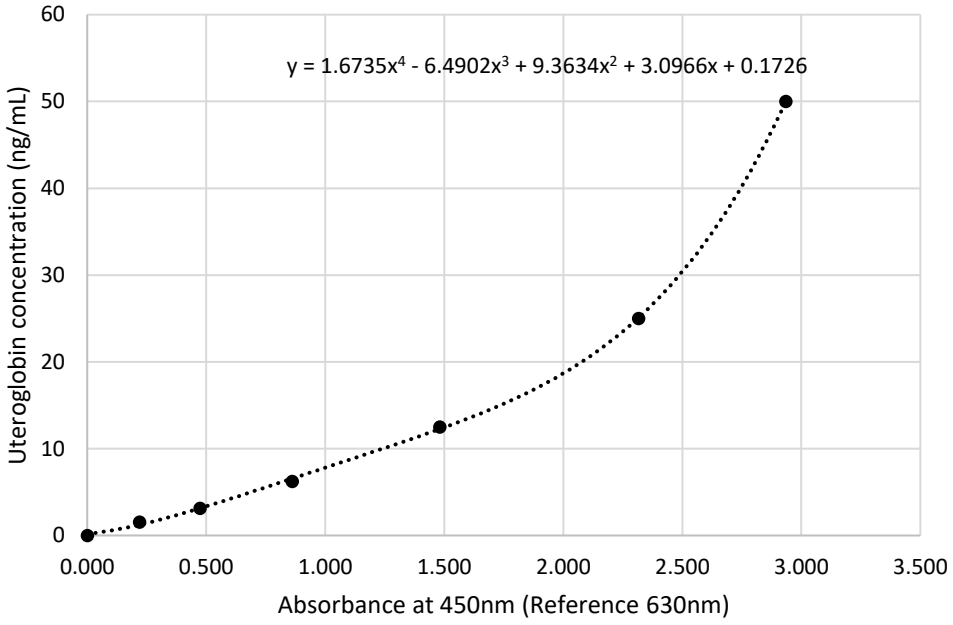


Figure 5.17: ELISA standard curve of uteroglobin concentration against optical density with curve fitting using the polynomial line of best fit

The equation of the line of best fit is shown. $R^2 = 0.999$. The curve was generated in Microsoft Excel.

The high and low quality control samples provided by the manufacturer had expected concentrations of 16.6 (11.6-21.6) and 4.85 (3.40-6.31) ng/mL respectively. The concentrations obtained using the 4PL method were 15.0 and 3.96 respectively. The equivalent values for the polynomial line of best fit were 14.7 and 3.88.

The albumin-depleted plamsa samples were tested in duplicate. The absorbance of one of the remission samples from SRNS3 was considerably lower than the replicate and all other plasma samaples, and was similar to the 0 ng/mL control. This was considered an outlier and was excluded from further analysis. Single wells were used for the non-albumin-depleted plasma samples due to limited space on the initial ELISA plate.

Table 5.12 shows a comparison of the relative quantification of uteroglobin in paired relapse and remission samples by ELISA and proteomics.

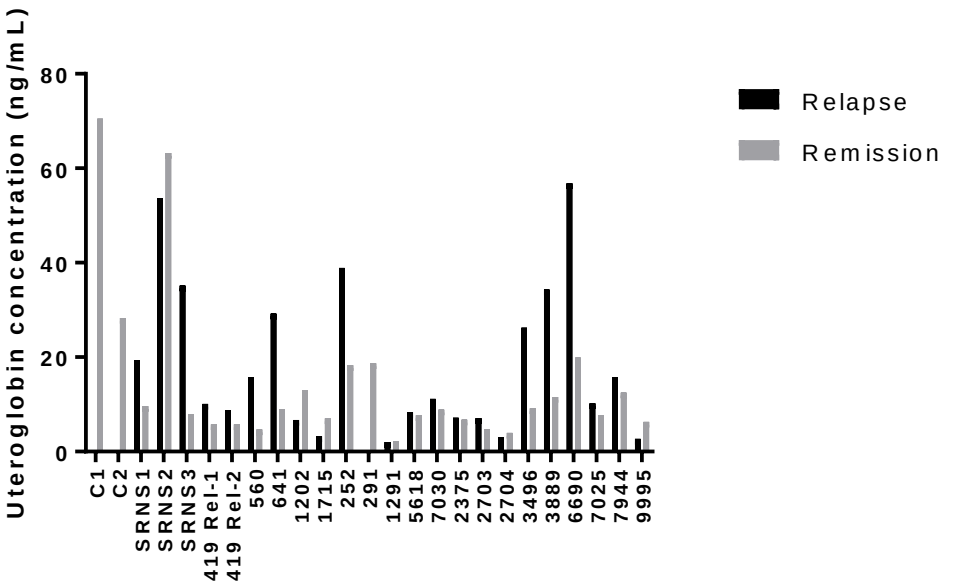
Table 5.12: Uteroglobin concentrations determined by ELISA in relapse and remission plasma samples from patients with SRNS

Patient plasma sample	Uteroglobin concentration (ng/mL)	ELISA (Rel/Rem ratio)	Proteomics (Rel/Rem ratio)
SRNS1 Rel AD	1.31	1.47	2.19
SRNS1 Rem AD	0.89		
SRNS2 Rel AD	3.42	0.82	2.03
SRNS2 Rem AD	4.15		
SRNS3 Rel AD	3.81	8.20	5.41
SRNS3 Rem AD	0.47		
SRNS3 Rel not AD	49.0	7.94	
SRNS3 Rem not AD	6.17		

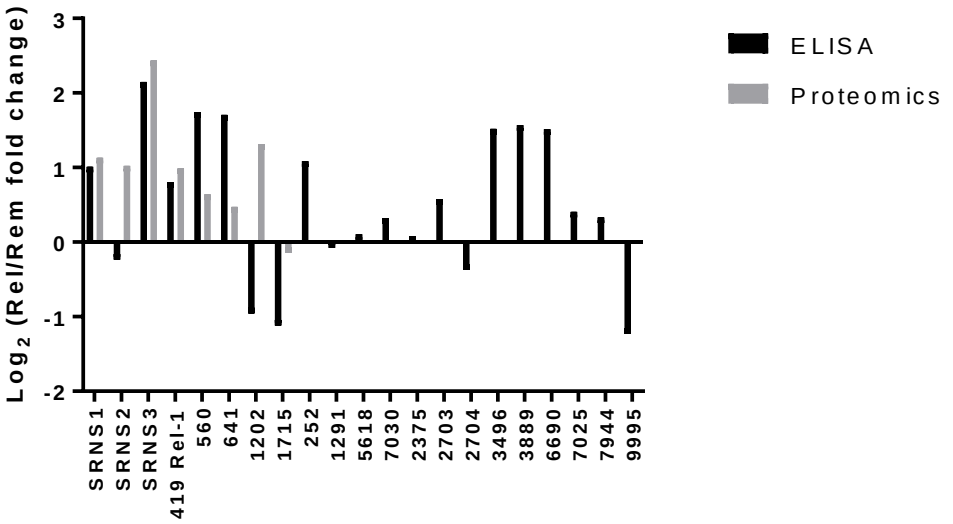
The concentrations were determined using the 4PL method.
Legend: AD, albumin-depleted; Rel, relapse; Rem, remission.

For patients SRNS1 and SRNS3, uteroglobin was higher in relapse than remission, consistent with proteomic findings. To allow direct comparison with proteomic results, plasma samples tested by ELISA were also albumin-depleted. The non-albumin-depleted plasma samples from SRNS3 had higher absolute concentrations of uteroglobin than the corresponding albumin-depleted samples. Importantly, however, the relapse/remission ratio was similar. On this basis, in the larger discovery cohort discussed below, plasma was not albumin-depleted prior to ELISA.

The uteroglobin concentrations in plasma samples from control subjects and paired relapse/remission samples from patients with NS are shown in Figure 5.18. The related clinic details were given previously in Table 5.5 and Table 5.6.



A



B

Patient	SRNS1	SRNS2	SRNS3	419	560	641	1202	1715
ELISA Rel/Rem fold change	2.02	0.85	4.43	1.74	3.35	3.27	0.52	0.46
Proteomics Rel/Rem fold change	2.19	2.03	5.41	1.99	1.56	1.39	2.49	0.90

C

Figure 5.18: ELISA and proteomic quantification of uteroglobin in paired relapse/remission plasma samples

A: Uteroglobin concentration in control (C1, C2) and paired relapse and remission plasma samples determined by ELISA. **B**: Log_2 transformed relapse/remission fold change ELISA quantification and MS proteomics. **C**: Relapse/remission fold change ratios determined from ELISA and MS.

Figure 5.18A shows the absolute concentration of uteroglobin in each of the samples tested. The results are data from two 96-well ELISA plates. The relapse sample for patient 291 had absorbance measurements similar to the 0 ng/mL control. This was considered to be an outlier and the patient excluded from further calculations. Although the 4PL method provided concentrations for all other samples tested, the validated range for the ELISA was 0-50 ng/mL, therefore results above 50 ng/mL should be treated with caution. Ideally these would be repeated at ½ dilution.

In this study it was evident that uteroglobin was present at relatively high concentrations in both control subjects C1 and C2 who had diseases other than SRNS (Figure 5.18A). The samples tested were both from PEx. As discussed previously (Section 5.3.5), uteroglobin is an immunomodulatory secretory protein which has been detected in a variety of body fluids and is increased in several conditions and, therefore, was not expected to be specific for NS. The absolute concentration of uteroglobin was variable in both relapse and remission within the group with SRNS (patients SRNS1-7030, in the left part of Figure 5.18A) and with SSNS (patients 2375-9995, in the right part of Figure 5.18A). However, across all 21 patients with SRNS or SSNS, the mean concentration of uteroglobin in relapse samples (18.9 ng/mL) was significantly higher than the mean concentration in remission samples (11.4 ng/mL, $p = 0.011$, paired t test, two tailed). For the 12 patients with SRNS, the concentrations were 19.5 ng/mL in relapse and 13.1 ng/mL in remission ($p = 0.084$, paired t test). The corresponding values for the 9 patients with SSNS were 13.1 ng/mL and 9.2 ng/mL respectively ($p = 0.084$, paired t test).

As shown in Figure 5.18B, the relapse/remission ratio was > 1.3 (> 0.38 on $\log_2$ scale) in 11 (52.4%) patients and < 0.77 (< -0.38 on $\log_2$ scale) in 3 (14.3%) patients. Comparison of findings by ELISA and MS proteomics showed close agreement for 3 patients in the first cohort (SRNS1, SRNS3 and 419) and fold changes in the same direction for 3 others (560, 641 and 1715). There were discordant results for SRNS2 and 1202. The remission plasma from the latter was derived from a blood sample whereas all other samples used in MS were from PEx. This lack of consistency may make comparison with the other patients more difficult but since the same samples were used for both ELISA and MS, this does would not directly explain the discordance.

The existence of any relationship between plasma uteroglobin concentration and degree of proteinuria was examined. A uPCR measurement was available on the same day (or within a few days) of the tested plasma sample for 18 patients (34 samples, 19 at time of "relapse" and 15 at time of "remission"). Samples for which there were no uPCR data (including those with only urine dipstick or ACR) were excluded. Figure 5.19 shows uteroglobin concentration plotted against $\log_{10}$ uPCR.

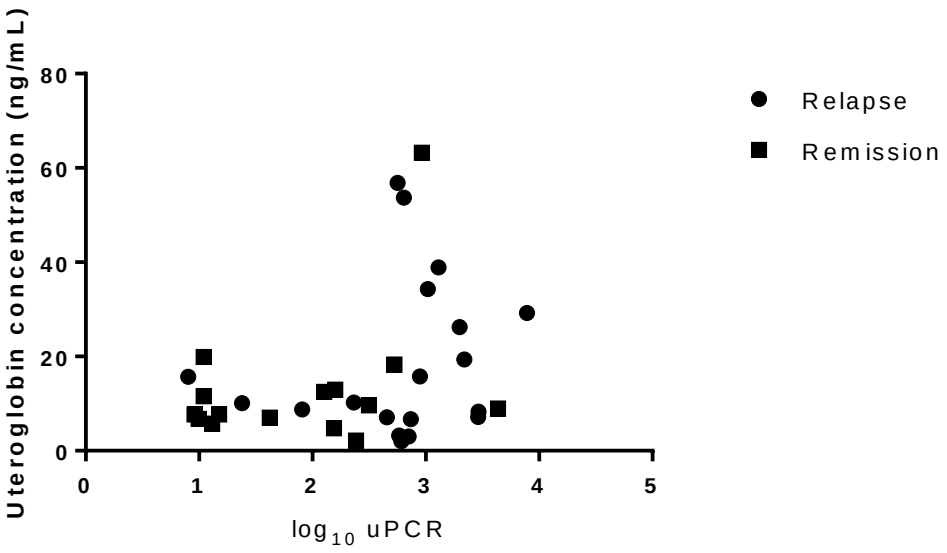


Figure 5.19: Relationship of uteroglobin concentration in plasma to uPCR for samples taken during relapse and remission in patients with SRNS and SSNS

There was no significant correlation between the two for relapse samples (Spearman r = 0.19, p = 0.44) or for remission samples (Spearman r = 0.26, p = 0.34). When all 34 samples were considered together there was also no significant correlation between uteroglobin and $\log_{10}$ uPCR (Spearman r = 0.26, p = 0.13).

5.3.8 Validation of mass spectrometry proteomics findings for lumican by ELISA

ELISA was used to quantify lumican in patient plasma as an alternative assay to Western blotting as discussed above (Section 5.3.6). Literature was reviewed to determine the normal concentration of lumican in plasma of healthy subjects which was reported as 4000 ng/mL or 7881 ng/mL [329, 341]. On this basis,

samples were diluted to the working range of the ELISA (0.313-20 ng/mL, Elabscience, E-EL-H0198). However, this resulted in quantities below the limit of detection. Importantly, the reports of plasma lumican concentrations over 4000 ng/mL were based on MS quantification data. A subsequent and more detailed literature search identified a study which had used ELISA to measure plasma lumican concentrations and reported 0.85 ± 0.53 ng/mL as the mean ± SEM in normal healthy controls [342].

Non-albumin-depleted plasma samples were used for the lumican ELISA after confirming a similar relapse/remission ratio for albumin-depleted samples for SRNS3 as previously seen for uteroglobin. The polynomial line of best fit was used for the standard curve as recommended by the ELISA manufacturer (Figure 5.20).

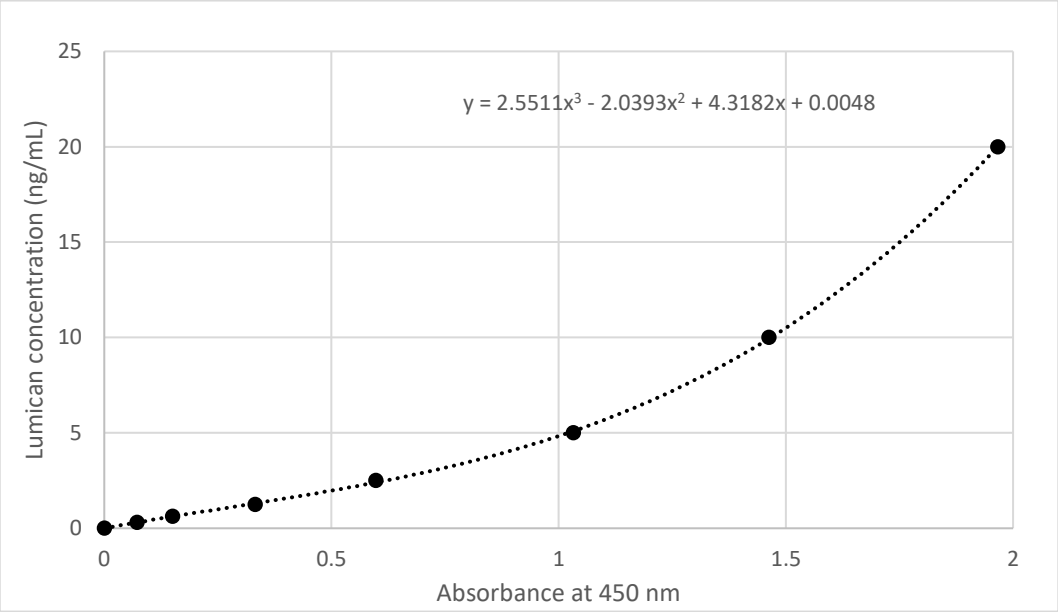
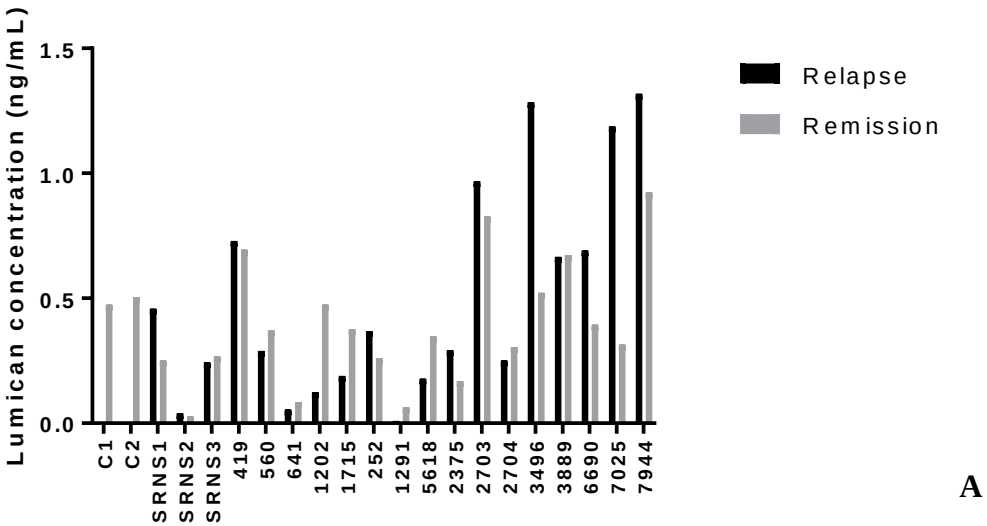
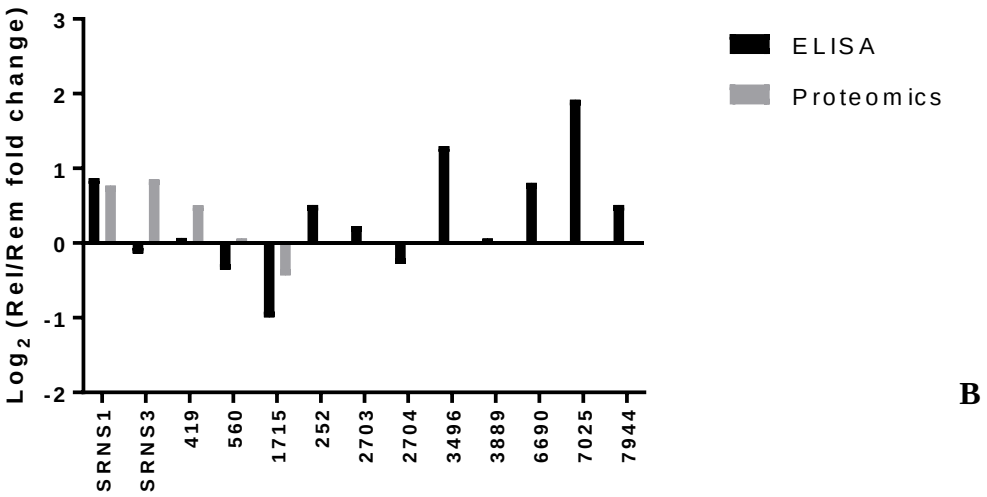


Figure 5.20: ELISA standard curve of lumican concentration against absorbance with curve fitting using the polynomial line of best fit

The equation of the line of best fit is shown. $R^2 = 0.999$. The curve was generated in Microsoft Excel.



A



B

Patient	SRNS1	SRNS2	SRNS3	419	560	641	1202	1715
ELISA Rel/Rem fold change	1.83	N/A	0.91	1.05	0.78	N/A	N/A	0.50
Proteomics Rel/Rem fold change	1.71	1.36	1.80	1.42	0.99	2.26	1.40	0.74

C

Figure 5.21: ELISA and proteomic quantification of lumican in paired relapse/remission plasma samples

A: Lumican concentration in control (C1, C2) and paired relapse and remission plasma samples determined by ELISA. **B**: Log_2 transformed relapse/remission fold change ELISA quantification and MS proteomics. **C**: Relapse/remission fold change ratios determined from ELISA and MS. N/A: data not available because concentration below level of detection in at least one sample.

Figure 5.21A shows the absolute concentration of lumican in each of the samples tested. The minimum concentration detectable by the ELISA according to manufacturer's instructions was 0.188 ng/mL. All samples had a concentration at the lower end of the detectable range below 1.4 ng/mL. The following samples had a calculated lumican concentration below the minimal detectable by the assay: both samples from patients SRNS2, 641 and 1291; relapse samples from 1202 and 5618; and the remission sample from 2375. These 6 patients were therefore excluded, leaving 13 in the subsequent analysis.

Both control subjects C1 and C2 had similar lumican concentrations of 0.47 and 0.50 ng/mL respectively. Across the 13 patients with SRNS or SSNS, the concentration of lumican in relapse samples (mean 0.66, SD 0.41 ng/mL) was higher than in remission samples (mean 0.48, SD 0.23 ng/mL) although this was not statistically significant ($p = 0.055$, paired t test). For the 6 patients with SRNS, the concentrations were 0.38 ng/mL (SD 0.20) in relapse and 0.37 ng/mL (SD 0.17) in remission ($p = 0.87$, paired t test). Among the 7 patients with SSNS, the lumican concentration was significantly higher in relapse (mean 0.91 ng/mL, SD 0.39) than in remission (mean 0.57 ng/mL, SD 0.25, $p = 0.045$, paired t test).

Figure 5.21B illustrates that for patients with SSNS (2703-7944, in the right part of the figure), the relapse/remission ratio was > 1.3 (> 0.38 on $\log_2$ scale) in 4 (57.1%) of 7 patients. There were no consistent findings for the patients with SRNS (SRNS1-252, in the left part of the figure) and moderate agreement between ELISA and MS proteomics for only two of the patients (Figure 5.21C).

The existence of any relationship between the plasma lumican concentration and degree of proteinuria was examined. As for the uteroglobin

analysis, only samples for which an associated uPCR was available were included. Figure 5.22 shows the lumican concentration plotted against $\log_{10}$ uPCR.

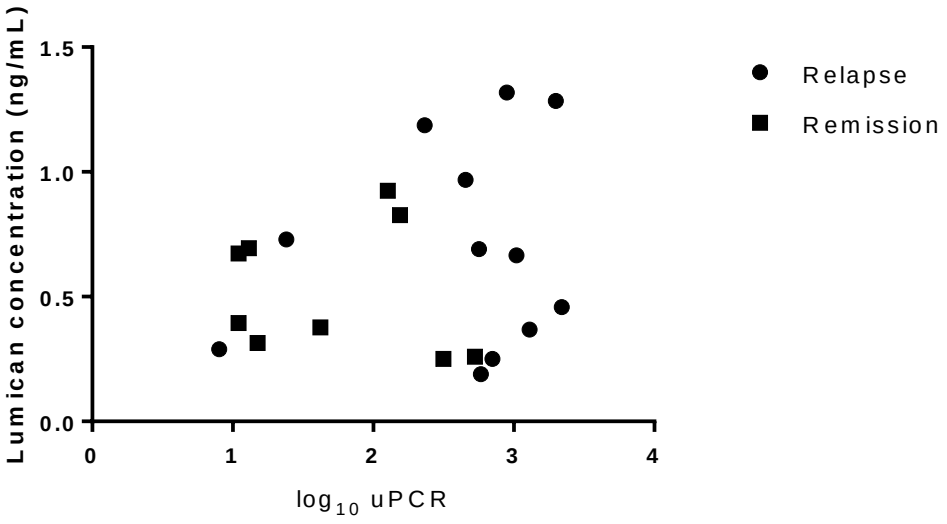


Figure 5.22: Relationship of lumican concentration in plasma to uPCR for samples taken during relapse and remission in patients with SRNS and SSNS

There was no significant correlation between the lumican concentration and uPCR for relapse samples (Spearman r = 0.028, p = 0.94) and a weak, non-significant, negative correlation for the remission samples (Spearman r = -0.33, p = 0.39). When all 21 samples were considered together there was no significant correlation (Spearman r = 0.063, p = 0.78).

5.4 Discussion

5.4.1 Findings of this study

This study set out to identify proteins which were differentially present in plasma of patients with NS at times of relapse and remission.

Analysis of MS quantification ratios using ordinary t tests identified no proteins which were significantly different in relapse versus remission plasma after correction for multiple comparisons (using either the Bonferroni correction or BH procedure). This was likely due to the large variance in the data with a small number of samples (4 patient pairs in the initial analysis) [356]. The application of Bayes moderated t tests, which are potentially more suited to analysis of proteomic data with small numbers of samples, also did not identify proteins with a statistically significant difference [320]. It is recognised that the Bonferroni correction, in particular, is very conservative and while minimising false positives also leads to false negatives; therefore, it may not be appropriate at the discovery phase of proteomic data analysis [357]. The use of fold-change thresholds and maximising consistency across subjects was employed as the main method for identifying potential biomarkers for further validation. Although this did not provide a measure of statistical significance for differences in proteins between relapse and remission, by using a minimum threshold of 1.3-fold it was felt that this would represent biological significance [358].

Using this methodology, with paired relapse and remission samples from 4 patients with SRNS and 5 patients with SSNS, identified 14 proteins with fold change > 1.3 and consistent across ≥ 7 patients. The top 6 proteins taken forward for further analysis were: Dystroglycan 1, EphB4, Lumican, Nek3, RAN binding protein 3 and uteroglobin. Although these were potentially promising candidates,

none could be validated convincingly using Western blotting. There was also a lack of consistency in the MS relative quantification data for the first set of 4 patients (the initial discovery cohort) and the second set of 4. Although all 8 patients had SRNS, had been transplanted and suffered post-transplant recurrence, it is not clear whether differences were biological or related to batch effects from being processed and run on the mass spectrometer at different times.

Based on biological information about the 6 proteins originally identified in the discovery cohort and published data of potential as biomarkers in other conditions, lumican and uteroglobin were taken forward for further analysis by ELISA. For lumican, 2 of the second cohort of 4 patients (641 and 1202) showed consistency on MS proteomics with fold-change > 1.3. The findings were not, however, corroborated on ELISA for the 8 patients with MS data. There was closer agreement between fold-change data from ELISA and MS proteomics for uteroglobin and across all 21 patients tested the concentration in relapse samples was significantly higher than in remission.

Taking MS and ELISA data together highlights uteroglobin as a potential biomarker worthy of further investigation in a larger cohort of patients. It is known that the protein is steroid-inducible [349], therefore the apparently higher concentration in relapse samples in this study may be the result of treatments patients were receiving. A future, prospective and longitudinal study would ideally obtain initial samples before any treatment is started, then subsequently on treatment at times of relapse and remission. This would help to address the question of whether uteroglobin is predictive of relapse or post-transplant recurrence which the current study is unable to answer.

5.4.2 Comparison of potential biomarkers from literature with proteomic data

As discussed in Chapter 1, previous studies have quantified and compared protein levels in subgroups of patients with NS to assess their potential as biomarkers. The most frequently-tested biological sample has been urine and comparisons made between MCD versus FSGS and SSNS versus SRNS (see Table 1.13 in Chapter 1). Only one recent publication, by Andersen *et al.*[257], has undertaken proteomic analysis of plasma samples and compared at times of NS relapse and remission, and is, therefore, more directly comparable with the current study. They did, however, use plasma derived from centrifugation of blood rather than plasma exchange samples, and only 3 of the patients had SRNS whereas the other 11 had SSNS. They identified 11 proteins which were significantly increased in plasma at the time of active disease and 6 proteins (excluding albumin) which were decreased. The comparison with proteomic data for 8 patients with SRNS in this study is shown in Table 5.13.

Table 5.13: Plasma proteins significantly altered in relapse versus remission in study by Andersen *et al* (2012): comparison with proteomic data from 8 patients with SRNS

Protein name	Gene name	Mean Rel/Rem ratio published*	SRNS1	SRNS2	SRNS3	419	560	641	1202	1715	n > 1.3	n < 0.77
Haptoglobin-related protein	HPR	3.3	1.12	0.77	0.89	1.10	1.02	1.93	0.60	0.82	1	1
Apolipoprotein B-100	APOB	3.2	1.82	0.61	1.22	1.11	0.89	1.35	0.93	0.71	2	2
Haptoglobin	HP	2.9	0.82	1.26	0.25	1.25	0.98	1.47	0.98	1.47	2	1
Inter-alpha-trypsin heavy chain H3	ITIH3	2.5	1.12	0.80	1.48	1.22	0.95	1.74	0.94	1.04	2	0
Alpha-2-macroglobulin	A2M	2.3	1.66	1.16	3.62	1.22	1.38	2.87	0.65	0.89	4	1
Fibrinogen beta chain	FGB	2.2	0.90	0.64	1.20	1.40	1.13	1.42	11.64†	0.62	3	2
Protein AMBP	AMBP	2.2	1.72	1.49	1.90	1.21	1.25	0.98	1.46	0.98	4	0
Fibrinogen gamma chain	FGG	2.1	0.83	0.82	1.34	1.30	1.14	1.21	8.28†	0.62	2	1
Fibrinogen alpha chain	FGA	2.0	0.85	0.65	1.27	1.41	1.11	1.37	8.53†	0.57	3	2
Pregnancy zone protein	PZP	2.0	2.03	0.72	1.00	1.19	0.73	1.33	0.55	0.56	2	4
Immunoglobulin J chain	IGJ	1.6	1.41	1.23	2.45	1.18	1.23	2.75	0.91	0.87	3	0
Alpha-1-acid glycoprotein 1	ORM1	0.6	0.73	0.88	0.24	1.20	0.76	0.66	0.96	1.32	1	4
Ig gamma-4 chain C region	IGHG4	0.5	No data									
Hemopexin	HPX	0.5	0.91	1.01	0.46	1.17	0.70	0.48	0.89	1.20	0	3
Ig gamma-3 chain C region	IGHG3	0.4	2.67	1.05	0.77	0.52	0.82	4.37	1.59	1.29	3	2
Serotransferrin	TF	0.4	1.47	1.12	0.93	1.29	0.37	No data	0.42	0.64	1	3
Ig gamma-1 chain C region	IGHG1	0.4	1.50	0.77	1.07	1.11	No data				1	1

Red highlights fold change > 1.3 and blue < 0.77. * Data from Andersen *et al*.[257] †Relapse sample from PEx, remission sample from blood

Overall, there was not strong consistency between the current study patients and the published relapse/remission ratios. Among the 11 proteins which were increased in active disease, in only a maximum of 4 patients was the relapse/remission ratio > 1.3, namely for protein AMBP and alpha-2-macroglobulin. The latter, however, appeared to be decreased in relapse (<0.77) in one patient. Protein AMBP, also known as Alpha-1-Microglobulin/Bikunin Precursor, is cleaved into alpha-1-microglobulin, inter-alpha-trypsin inhibitor light chain and trypstatin and functions include serine protease inhibitor activity [334]. As discussed in Chapter 1 (Section 1.4.3), proteases or protease inhibitors may have a role in SRNS pathogenesis but protein AMBP has not been associated with this disease in the published literature.

Looking at proteins which were decreased in plasma in active disease according to the study by Andersen *et al*., there were no quantification data available from the proteomic analysis of samples from some patients, particularly for immunoglobulins which had been depleted from the plasma as part of the preparation process. Where data were available, the greatest consistency in fold change was seen for alpha-1-acid glycoprotein 1 (AGP1) and hemopexin. The former, also known as orosomucoid-1, is an acute phase plasma protein which is increased in inflammation [334]. It is thought to have inflammatory and immunomodulatory functions with plasma levels altered in a wide range of diseases [359]. In a different study, which undertook proteomic analysis of urine comparing patients with SSNS versus SRNS, initial exploratory analysis suggested AGP1 was significantly increased in urine of subjects with SRNS but this difference was not significant in the larger validation cohort [255].

Hemopexin was decreased < 0.77-fold in relapse plasma in 3 patients in this study and in no patients was it increased > 1.3-fold. Other research by Bakker *et al*. found that hemopexin was reduced in plasma of patients with MCD at relapse compared with remission (0.21±0.14 mg/ml vs 0.44±0.06 mg/ml; p <0.01) [158]. The same group, using kidney tissue *in vitro*, showed increased protease activity of hemopexin in MCD relapse plasma compared with remission. Previous work at the University of Bristol examined the effect of hemopexin on conditionally-immortalised human podocytes *in vitro* [173]. This demonstrated that treatment of the cells with hemopexin led to actin cytoskeletal reorganisation and that the effect was dependent on nephrin and reduced by pre-incubation with normal human plasma.

A number of recent biomarker discovery studies in NS have used urine samples to identify proteins which are significantly different between disease subgroups. Although the methodologies and results are not directly comparable with the current study, the highlighted proteins were used in an exploratory analysis of the proteomic data from the 8 patients with SRNS at relapse and remission. The 22 proteins with corresponding relapse/remission ratios from this study are shown in Table 5.14.

Table 5.14: Plasma protein release/remission ratios for 8 patients with SRNS for proteins suggested as biomarkers in the published literature

Publication (first author, year, reference)	**Groups compared**	**Protein name**	**Gene name**	**SRNS1**	**SRNS2**	**SRNS3**	**419**	**560**	**641**	**1202**	**1715**	**n > 1.3**	**n < 0.77**
Pérez 2017 [236]	MCD vs FSGS	Alpha-1 antitrypsin (AAT)	SERPINA1	0.64	0.98	0.65	1.19	0.83	0.94	1.06	1.30	1	2
Pérez 2017 [236]	MCD vs FSGS	Serotransferrin	TF	1.47	1.12	0.93	1.29	0.37	No data	0.42	0.64	1	3
Pérez 2017 [236]	MCD vs FSGS	Histatin-3	HTN-3	No data									
Pérez 2017 [236]	MCD vs FSGS	39S ribosomal protein L17, mitochondrial	MRPL17	No data									
Choi 2017 [237]	MCD vs FSGS	Complement component 9	C9	0.60	0.71	0.48	1.31	0.87	0.86	0.57	1.08	1	4
Choi 2017 [237]	MCD vs FSGS	Monocyte differentiation antigen CD14	CD14	0.82	0.85	0.73	1.17	0.78	1.58	1.06	0.84	1	1
Nafar 2014 [244]	FSGS vs controls	CD59 glycoprotein	CD59	2.05	1.89	4.16	1.06	1.40	1.01	2.58	0.92	5	0
Nafar 2014 [244]	FSGS vs controls	CD44 antigen	CD44	1.99	0.99	1.16	1.14	1.05	3.43	1.29	0.79	2	0

Nafar 2014 [244]	FSGS vs controls	Insulin-like growth factor-binding protein 7	IGFBP7	1.22	1.43	2.53	1.51	1.03	1.19	1.60	0.97	4	0
Nafar 2014 [244]	FSGS vs controls	Roundabout homolog 4	ROBO4	1.19	0.96	1.02	1.10	1.03	2.76	1.06	0.79	1	0
Nafar 2014 [244]	FSGS vs controls	Dipeptidase 1	DPEP1	No data									
Suresh 2016 [246]	SSNS vs SRNS	Apolipoprotein A-I	APOA1	1.60	0.78	0.95	1.21	0.88	0.98	0.93	0.78	1	0
Suresh 2016 [246]	SRNS-MCD vs SRNS-FSGS	Alpha-2 macroglobulin	A2M	1.66	1.16	3.62	1.22	1.38	2.87	0.65	0.89	4	1
Suresh 2016 [246]	SRNS-MCD vs SRNS-FSGS	Alpha-1-acid glycoprotein 2 (Orosomucoid-2)	ORM2	0.83	0.78	0.28	1.23	0.71	0.64	0.82	1.03	0	3
Suresh 2016 [246]	SRNS-MCD vs SRNS-FSGS	Retinol binding protein 4	RBP4	1.58	1.31	0.90	1.01	0.99	0.71	1.44	0.79	3	1
Kalantari 2014 [247]	SSNS vs SRNS	Matrix remodelling-associated protein 8	MXRA8	1.35	0.68	0.20	1.88					2	2
Nickavar 2016 [248], Bennett 2017 [255]	SSNS vs SRNS	Neutrophil gelatinase-associated lipocalin (NGAL)	LCN2	2.60	0.85	3.00	1.50	1.43	2.38	1.37	2.03	7	0
Bennett 2016 [250], Bennett 2017 [255]	SSNS vs SRNS	Vitamin D-binding protein	GC	0.70	0.92	0.53	1.06	0.72	0.59	0.87	0.76	0	5

Bennett 2017 [255]	SSNS vs SRNS	Transthyretin (Prealbumin)	TTR	1.66	1.26	0.79	1.25	0.76	0.35	1.82	1.29	2	2
Bennett 2017 [255]	SSNS vs SRNS	Alpha-2-HS-glycoprotein (Fetuin A)	AHSG	0.59	0.56	0.56	1.04	0.68	1.04	0.52	0.57	0	6
Watany 2018 [256]	SSNS vs SRNS	Nephronectin	NPNT	No data									
Andersen 2012 [257]	Active NS vs remission	Cadherin-1	CDH1	2.09	0.85	1.63	1.48	1.16	2.52	1.16	0.91	4	0

Four proteins were not detected or were not quantifiable in plasma in this study, therefore had no data for analysis. Among the rest, there was variability in the relapse/remission ratio between the patients but the protein showing the most consistent increase in plasma greater than 1.3-fold in relapse was neutrophil gelatinase-associated lipocalin (NGAL). It was above this threshold in 7 of the 8 patients studied and increased above 2-fold in 4 of them.

NGAL is an iron-binding protein with roles in multiple processes including regulation of apoptosis, innate immunity and nephrogenesis [360]. Two recent publications measuring urinary NGAL in patients with NS found it was significantly higher in those with SRNS compared with SSNS [248, 255]. A growing body of evidence suggests that both plasma and urinary NGAL are clinically-useful biomarkers of early acute kidney injury in adults and children [360-362]. The wide range of clinical contexts associated with elevated NGAL, including sepsis, post-cardiac surgery, haemolytic uraemic syndrome and urinary tract infection, indicates that it is not specific for disease relapse in SRNS. It may be a protein worthy of further study longitudinally in the context of children with SRNS before and after transplantation.

Of the proteins identified in the literature as possible biomarkers, the one most consistently decreased under 0.77-fold in relapse in this proteomic study was alpha-2-HS-glycoprotein (AHSG), also known as fetuin A. It was reduced in relapse below the threshold in 6 of the 8 patients. AHSG is mainly synthesised by the liver and has diverse roles including anti-inflammatory properties, regulation of calcium metabolism and bone remodelling, and insulin resistance [363, 364]. Bennett *et al*. showed that urinary AHSG was significantly higher in patients with SRNS compared with SSNS [255]. Two other studies suggested that AHSG was

lower in the plasma of patients with NS in relapse compared with those in remission or controls [365, 366]. The level of AHSG correlated with serum albumin and negatively correlated with proteinuria suggesting that the reduction in relapse may be simply due to loss in urine. Whether AHSG offers any advantages as a biomarker over measurement of plasma albumin and uPCR would require prospective longitudinal study in a larger cohort of patients with SRNS.

5.4.3 Limitations

This study has several limitations. As is often the case with MS proteomic investigations, the sample size in the initial discovery cohort was relatively small [320]. As the research progressed, additional paired relapse and remission samples were received as part of the NephroS study which were used in the subsequent validation cohort. Attempts were made to select an initial group of patients who were as similar as possible: all with SRNS and post-transplant recurrence of disease suggesting a circulating factor pathogenesis. Despite this, the pattern of initial steroid resistance and first biopsy findings differed within the 4 patients in the discovery cohort. Whether the same circulating factor(s) were involved in pathogenesis in these or subsequent patients is unknown. The degree of proteinuria associated with samples labelled as "relapse" varied between patients, and comparator samples from some were only available at times of partial remission since full remission was never achieved. The drug treatments received are likely to have varied between patients and within the same patient at different times depending on disease status: this has the potential to confound biomarker detection if the plasma protein levels are affected directly by the drug [228].

Differences in sample handling may have contributed to variability in relapse/remission ratios between patients. Plasma exchange fluid was stored at local centres at 4°C, sometimes for several days, before transport to the central centre in cool bags with ice packs and aliquoted on the day of arrival for storage at -80°C. Blood samples (taken at remission for patient 1202 and other patients in the wider validation cohort) were transported at ambient temperature by standard post often arriving for processing > 24 hours after leaving the patient. It is possible that some alteration or degradation of proteins within the samples would have taken place potentially affecting results of this study.

In addition to patient and sample factors, some variability in the protein quantification may have resulted from the sample preparation and MS process. Although the same process of albumin and Ig depletion, TMT labelling and nano-LC-MS/MS were performed for all samples, there is natural variation in the efficacy of depletion and labelling which may depend on the biophysical properties of different plasma samples and so cannot be completely controlled. Also, due to the number and complexity of peptides in a sample, only a subset of all proteins is identified in a single MS experiment [367]. The use of TMT labelling and multiplexing in this study should have reduced some of the experimental variability in the MS quantification process.

5.4.4 Conclusion

This study has identified uteroglobin as a protein which is increased in relapse compared with remission in patients with SRNS. It has also indicated that hemopexin, NGAL and AHSG, which have previously been suggested as potential biomarkers in other studies, are differentially present in relapse and

remission plasma. Further analysis of these proteins in a larger cohort of patients with longitudinal sampling would help to determine their value as predictive indicators of disease.

The approach used in this study involved a non-targeted analysis of the whole plasma proteome using MS in a discovery cohort of patients followed by validation in a larger group using Western blotting and ELISA. A second approach specifically examining proteases and protease inhibitors, which may have a direct pathogenic role in NS, will be discussed in Chapter 6.

5.5 Appendix to Chapter 5

5.5.1 R code for empirical Bayes moderated t tests of quantitative mass spectrometry proteomics data

Modified from Kammers *et al*. [320] and publicly available to download from: http://www.biostat.jhsph.edu/~kkammers/software/eupa/

Install R from CRAN and the basic Bioconductor packages by typing the following code in your R command window

```
source("http://bioconductor.org/biocLite.R")
biocLite()
```

Now install additional Bioconductor packages. These packages are necessary for the proteomic data analysis. Make sure to install and update all dependencies when you are asked.

```
biocLite("limma")
biocLite("qvalue")
```

Load the following packages by typing in your R command window

```
library(limma)
library(qvalue)
# read artificial iTRAQ data set
dat <- read.csv("F:/Results/Proteomics results/PLAS_Total round 1/
Median normalised/Pooled 1-15 5FDR Exp bias Median normalised Pept
ide ungrouped raw quant data for R.csv")
dim(dat)
str(dat)
cha <- c("X127C", "X128C", "X129C", "X130C", "X128N", "X129N", "X1
30N", "X131")
# data preprocessing, load all functions
source("http://www.biostat.jhsph.edu/~kkammers/software/eupa/sourc
e.functions.r")
dat <- read.peptides(dat, cha)
dim(dat)
```

```
dat <- quantify.proteins(dat, cha)
```

```
dat.onehit <- subset(dat, dat$n.peptides == 1)
```

```
dim(dat.onehit)
```

```
# eliminate "one-hit wonders"
dat <- subset(dat, dat$n.peptides != 1)
```

```
dim(dat)
```

```
par(mfrow=c(1,1), font.lab=2, cex.lab=1.2, font.axis=2, cex.axis=1
.2)
boxplot(dat[, 1:length(cha)],  ylim = c(-3, 3), main="Boxplot norm
alized Intensities")
tr <- c("X127C", "X128C", "X129C", "X130C")
ct <- c("X128N", "X129N", "X130N", "X131")
design <- model.matrix(~factor(c(2,2,2,2,1,1,1,1)))
design
colnames(design) <- c("Intercept", "Diff")
res.eb <- eb.fit(dat[, c(tr,ct)], design)
head(res.eb)
# volcano plots for ordinary and moderated p-values
rx <- c(-1, 1)*max(abs(res.eb$logFC))*1.1
ry <- c(0, ceiling(max(-log10(res.eb$p.ord), -log10(res.eb$p.mod))
))


par(mfrow=c(1,2), font.lab=2, cex.lab=1.2, font.axis=2, cex.axis=1
.2)
par(las=1, xaxs="i", yaxs="i")


plot(res.eb$logFC, -log10(res.eb$p.ord), pch=21, bg="lightgrey", c
ex=0.9,
     xlim=rx, ylim=ry, xaxt="n",
     xlab="fold change", ylab="-log10  p-value")
abline(v=seq(-2,2,1), col="lightgray", lty="dotted")
abline(h=seq(0,ry[2],1), col="lightgray", lty="dotted")
axis(1, seq(-2,2,1), paste(c("1/4","1/2","1/1","2/1","4/1")))
title("volcano plot of ordinary p-values")


plot(res.eb$logFC, -log10(res.eb$p.mod), pch=21, bg="lightgrey", c
ex=0.9,
     xlim=rx, ylim=ry, xaxt="n",
```

```
     xlab="fold change", ylab="-log10  p-value")
abline(v=seq(-2,2,1), col="lightgray", lty="dotted")
abline(h=seq(0,6,1), col="lightgray", lty="dotted")
axis(1, seq(-2,2,1), paste(c("1/4","1/2","1/1","2/1","4/1")))
title("volcano plot of moderated p-values")
```

Chapter 6 Plasma Proteases and Protease Inhibitors

6.1 Introduction

As highlighted in Chapter 1 (Section 1.4.1), several strands of evidence point to the role of circulating factors within plasma as being pathogenic in non-genetic SRNS. Given previous studies suggesting that an increase / decrease in circulating proteases or protease inhibitors may be involved in the pathogenesis of SRNS [157, 158, 188], this part of the study sought to compare relative levels of 35 human proteases and 32 protease inhibitors in paired relapse and remission plasma samples.

6.2 Methods

6.2.1 Patients

Patients were selected for investigation from the cohort as described previously in Chapter 5.

6.2.2 Human protease/protease inhibitor array

The Human Protease/Protease Inhibitor Array Kit (R&D Systems, Minneapolis, MN, USA; #ARY025) was used following the manufacturer's instructions. Briefly, paired relapse and remission plasma samples were defrosted. They were incubated for 1 hour at room temperature in the supplied buffer with biotinylated detection antibody cocktails targeting proteases and separately targeting protease inhibitors. Nitrocellulose membranes were supplied spotted in duplicate with

control and capture antibodies against the 35 proteases or 32 protease inhibitors. The membranes were incubated in blocking buffer with agitation for 1 hour at room temperature. Following incubation, the plasma/biotinylated antibody mixtures were added to the respective membranes and left overnight at 4°C to allow binding. The following day, the membranes were washed 3 × 10 minutes with wash buffer and incubated with streptavidin-HRP in array buffer for 30 minutes at room temperature. After a further 3 × 10-minute washes, chemiluminescence reagents were applied for 1 minute and the membranes imaged simultaneously using the Amersham Imager 600 using the automatic capture settings.

Densitometry of each dot was undertaken with Image J. The mean intensities of the two negative control dots was subtracted from all other intensities to account for background. In the cases where a dot's intensity was lower than the negative control these were given an intensity of 0. In order to compare two membranes, one used for relapse plasma and the other for remission plasma, a normalisation factor was calculated: (mean intensity of all positive control dots on "relapse" membrane) / (mean intensity of all positive control dots on "remission" membrane). The intensities of all dots on the "remission" membrane were multiplied by this factor to account for global differences in the processing and imaging. In order to compare between all patients, a similar normalisation factor was calculated: (mean intensity of all positive control dots on SRNS1 "relapse" membrane) / (mean intensity of all positive control dots on "relapse" membrane for each patient). The intensities of all dots on the "relapse" membrane for each patient were multiplied by their respective normalisation factor. The same process was repeated for the "remission" membranes.

6.2.3 Statistical analysis

Data were analysed using Microsoft Excel 2016 and GraphPad Prism 7. The geometric mean was used to summarise fold change data across multiple patients. Statistical analysis was undertaken on the $\log_2$ transformed values. The paired t test (with two tails) was used to compare protein quantification data at the time of relapse and remission. The Bonferroni correction and Benjamini-Hochberg procedures were used to account for multiple comparisons as described previously. The association between variables was determined using Spearman's rank correlation where bivariate normal distributions could not be assumed. The threshold for rejecting the null hypothesis was set at $p < 0.05$.

6.3 Results

6.3.1 Patient characteristics

The following patients, whose samples were used previously for MS analysis, were included in this study: SRNS1, SRNS2, SRNS3, 560, 641, 1715. Other patients with SRNS (252, 1291 and 5618) and one patient with SSNS (2704) were also tested. The clinical characteristics and laboratory results at the time of sampling are shown in Table 5.5 and Table 5.6.

6.3.2 Protease and Protease inhibitor assays

The Human Protease/Protease Inhibitor Array Kit (R&D Systems) was used to quantify 35 proteases and 32 protease inhibitors in the paired relapse and

remission plasma samples for each of the 10 patients. An example of the dot array output generated from the assay is shown in Figure 6.1.

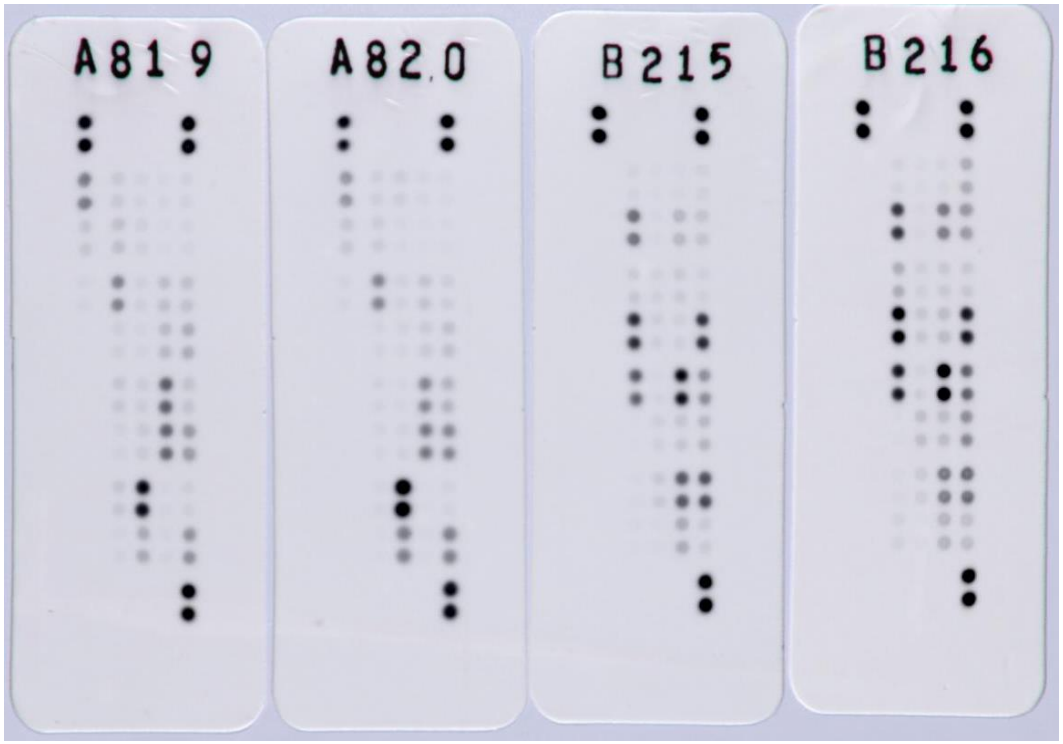


Figure 6.1: Dot array output from the Human Protease/Protease Inhibitor Array Kit for patient 1291

Legend: A819, proteases in relapse; A820, proteases in remission; B215, protease inhibitors in relapse; B216, protease inhibitors in remission

6.3.2.1 *Protease assays*

The log_2-transformed relapse/remission quantification ratios for proteases are shown in Figure 6.2.

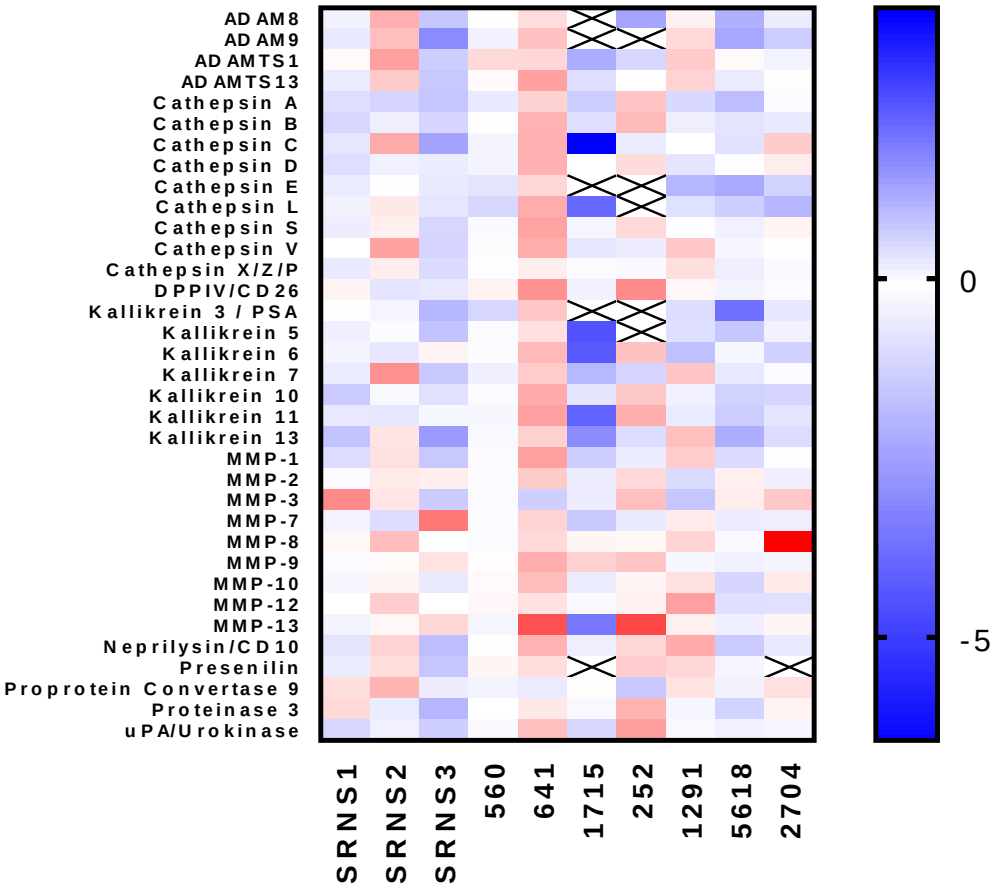


Figure 6.2: Heat map of log_2 (relapse/remission fold change) for proteases in plasma from patients with nephrotic syndrome

Data are shown for 35 proteases assayed in the Human Protease/Protease Inhibitor Array Kit (R&D Systems) and 10 patients. SRNS1-1715 (6 patients) had SRNS and were included in the MS proteomics studies. 252-5618 (3 patients) had SRNS and 2704 had SSNS. Proteases which are increased in relapse relative to remission are shown in red, those which are decreased are shown in blue. X denotes cases where the protease quantification for either the relapse or remission plasma sample was equivalent to the negative control and a fold change could not be calculated.

Overall, the heatmap illustrates a lack of a consistent pattern between the patients. For patient 560, for the majority of the proteases there was virtually no change between relapse and remission. This would concord with the available clinical data (see Table 5.6), namely that the samples were taken 9 days apart and both were, in fact, in remission (the sample labelled as "relapse" was associated with the nearest-available uPCR of 8 mg/mmol). Patient 641 had a particularly consistent increase in proteases in relapse compared to remission. In this case, the samples were approximately 1 month apart with plasma albumin and uPCR being 20 g/L and 7797 mg/mmol respectively at relapse and 35 g/L and 4348 mg/mmol at remission. SRNS2 and 252 both had comparatively more proteases increased in relapse relative to remission. All three of these patients had PSR, and although the plasma albumin was > 30 g/L at the time of the "remission" sample, they remained significantly proteinuric (uPCR > 500 mg/mmol). In contrast, in SRNS3, 1715 and 5618 the pattern was of proteases generally being decreased in relapse compared with remission. SRNS3 had SSR whereas the other two had PSR. Laboratory data were not available for SRNS3, however, for 1715 and 5618 the remission samples were obtained at a time of (almost) complete remission with uPCR values of 42 mg/mmol and 9.1 mg/mmol respectively.

Figure 6.3 illustrates the relationship between the geometric mean of all the relapse/remission fold change ratios for all 35 proteases and the relapse/remission uPCR ratio for the 7 patients in whom all uPCR values were available.

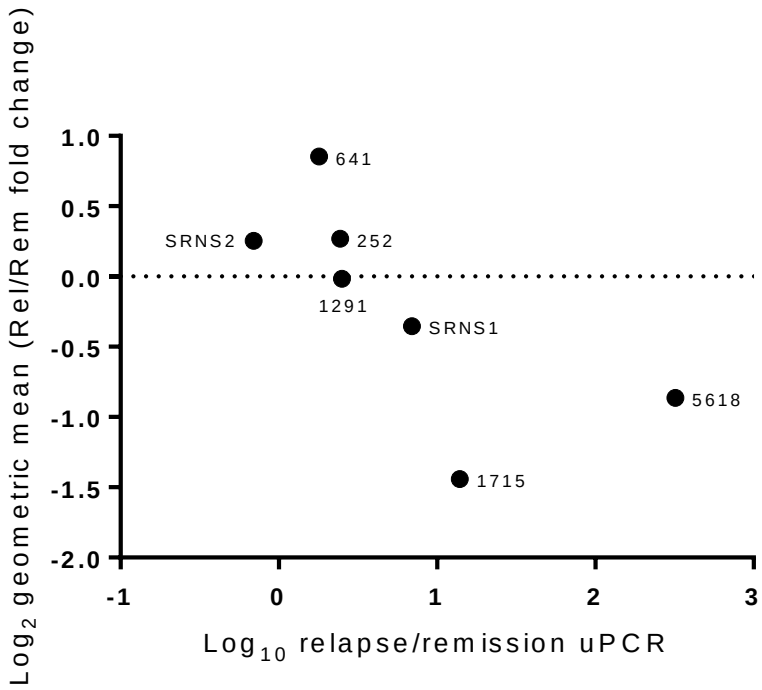


Figure 6.3: Relationship between the geometric mean of relapse/remission fold change ratio for all proteases and the relapse/remission uPCR ratio

There was a statistically significant, very strong negative correlation between the log-transformed variables (Spearman r = -0.86, p = 0.024). That is, patients with more severe relative proteinuria in relapse have comparatively lower proteases in relapse versus remission. The most logical explanation would be that, as might be expected, proteases are lost in the urine along with many other proteins during relapse. Although this may be the case in general, further investigations were undertaken to examine if any of the individual proteases were increased or decreased significantly and consistently across all patients.

As discussed previously in relation to MS proteomics (Section 5.3.3.1), the geometric mean relapse/remission fold change and t-test p values were calculated for each of the proteases measured in all 10 patients. The data are shown in the volcano plot (Figure 6.4).

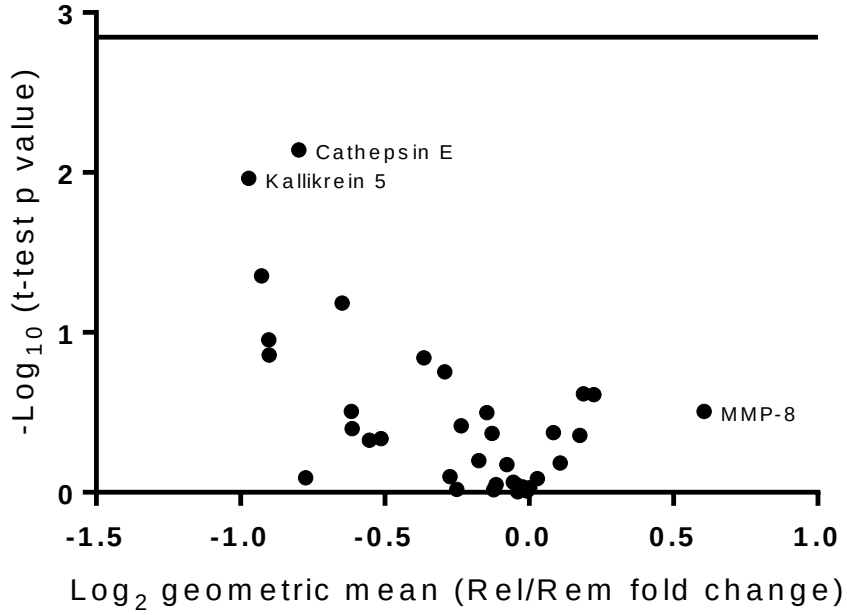


Figure 6.4: Volcano plot of protease quantification data for relapse/remission plasma samples from patients with nephrotic syndrome

The horizontal line shows the Bonferroni-corrected significance threshold for 35 proteins with $\alpha = 0.05$

It can be seen that cathepsin E and kallikrein 5 showed the most significant changes (with geometric mean relapse/remission fold changes of 0.58 and 0.51 respectively). However, no protease reached the Bonferroni-corrected significance threshold for multiple comparisons or were significant after the BH procedure with FDR at 10%.

An analysis using fold change thresholds of > 1.3 and < 0.77 with the aim of maximising consistency across patients is shown in Table 6.1. Although kallikrein 5 was a protein with one of the most significant fold changes, it was not in the top 7 proteins, as shown in the table, because it met the threshold (< 0.77) in only 5 of the 10 patients. Other proteins listed met the threshold in 6 or 7 patients.

Table 6.1: Proteases consistently increased or decreased in relapse versus remission across 10 patients with nephrotic syndrome

Target	SRNS1	SRNS2	SRNS3	560	641	1715	252	1291	5618	2704	n > 1.3	n < 0.77
MMP-8	1.10	1.96	1.01	0.91	1.47	1.10	1.10	1.56	0.89	13.62	4	0
MMP-9	0.92	1.04	1.34	1.01	2.30	1.61	1.80	0.87	0.77	0.82	4	0
Presenilin	0.72	1.42	0.36	1.11	1.41	N/A	1.66	1.50	0.83	N/A	4	2
ADAMTS1	1.06	2.67	0.41	1.48	1.50	0.23	0.49	1.71	1.05	0.80	4	3
Proprotein	1.39	2.15	0.72	0.80	0.70	1.03	0.38	1.35	0.79	1.37	4	3
MMP-3	3.38	1.30	0.40	0.93	0.43	0.72	1.92	0.37	1.20	1.76	4	4
Neprilysin/CD10	0.62	1.56	0.32	0.97	2.19	0.73	1.51	2.35	0.39	0.67	4	5
Cathepsin A	0.56	0.47	0.36	0.68	1.57	0.41	1.84	0.51	0.31	0.92	2	7
Cathepsin B	0.48	0.73	0.47	1.01	2.20	0.57	2.02	0.73	0.62	0.68	2	7
Cathepsin E	0.69	0.98	0.68	0.61	1.51	N/A	N/A	0.28	0.22	0.45	1	6
Cathepsin L	0.79	1.26	0.64	0.51	2.33	0.07	N/A	0.57	0.43	0.28	1	6
Kallikrein 11	0.67	0.64	0.85	0.87	2.66	0.06	2.29	0.69	0.41	0.63	2	6
Kallikrein 7	0.69	3.09	0.38	0.75	1.70	0.29	0.47	1.80	0.66	0.92	3	6
Kallikrein 13	0.35	1.31	0.17	0.88	1.61	0.13	0.53	1.90	0.24	0.54	3	6

Red highlights fold change > 1.3 and blue < 0.77. Patient 2704 had SSNS, all others had SRNS. The top 7 consistently increased and decreased proteases are shown.

The results for patient 641 appeared inconsistent with others, suggesting it may have been an outlier. Among the proteins which were generally decreased during relapse in most patients (with fold change < 0.77, in the lower part of the table), in all cases they were increased in relapse in 641. This may have been because there was still heavy proteinuria at the time the (partial) remission sample was obtained (uPCR 4348 mg/mmol at remission and 7797 mg/mmol at relapse).

For the 35 proteases analysed, the median molecular weight was 52 kDa (range 27 – 154 kDa). There was a moderate positive correlation between protease molecular weight and $\log_2$ geometric mean of the relapse/remission fold change (Spearman r = 0.40, p = 0.017). That is, smaller proteases were relatively lower in relapse than remission compared with larger proteases.

As discussed above, due to general loss of proteins in urine at relapse, it is possible that those proteases which are relatively increased in relapse are ones which may have potential as biomarkers or provide insight into pathogenesis. Taking MMP-8 as an example, however, it was most increased in the SRNS patients (SRNS2, 641 and 1291) who had least relative proteinuria in relapse (Figure 6.3). It was particularly increased in 2704 who had SSNS. In this patient, no uPCR was available at the time of remission. The relapse sample was taken approximately 6 months later with plasma albumin 28 g/L and uPCR 704 mg/mmol.

As discussed in Chapter 1, suPAR has been proposed as having a possible pathogenic role in SRNS. The protease array included detection of urokinase-type plasminogen activator (uPA). The relapse/remission ratios for this protease are shown in Table 6.2.

Table 6.2: Relapse/remission ratio of urokinase-type plasminogen activator in patients with nephrotic syndrome

	SRNS1	SRNS2	SRNS3	560	641	1715	252	1291	5618	2704
uPA	0.49	0.76	0.43	0.88	1.92	0.48	2.67	0.88	0.75	0.83

Red highlights fold change > 1.3 and blue < 0.77. Patient 2704 had SSNS, all others had SRNS.

In most patients, uPA was decreased in relapse but was increased in those patients with least relative proteinuria at the time of relapse.

6.3.2.2 Protease inhibitor assays

The $\log_2$-transformed relapse/remission quantification ratios for 32 protease inhibitors are shown in Figure 6.5.

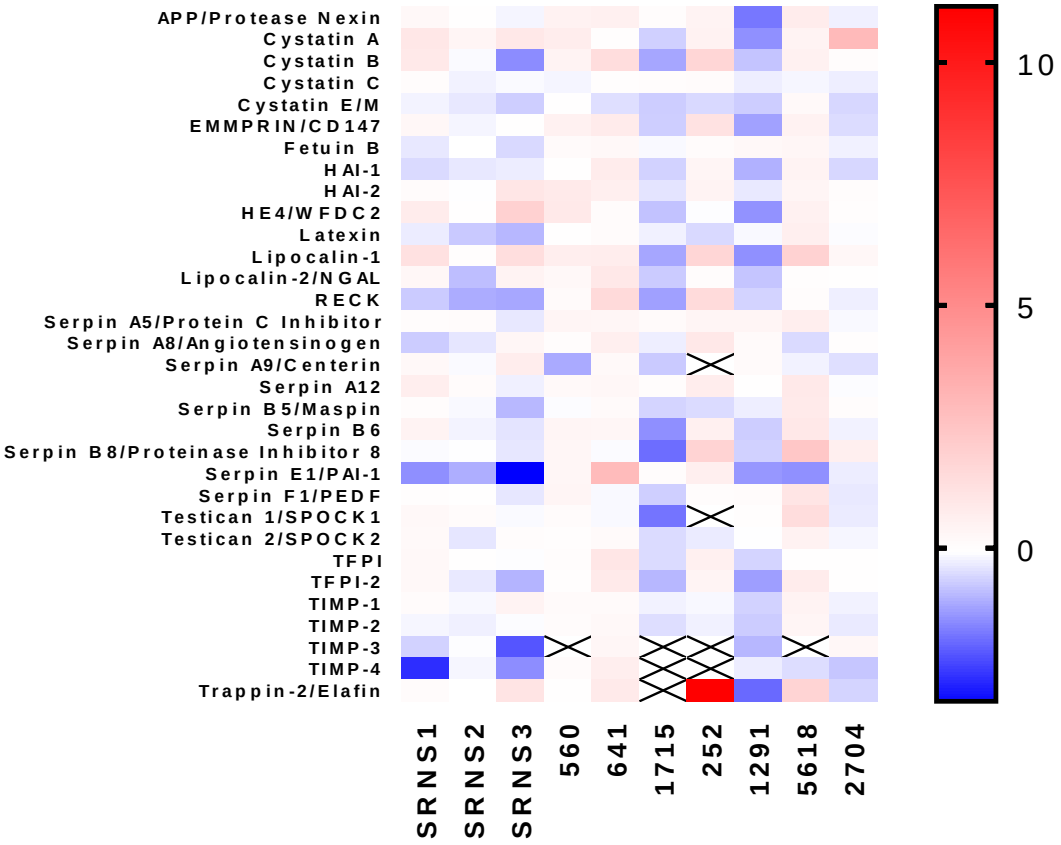


Figure 6.5: Heat map of $\log_2$ (relapse/remission fold change) for protease inhibitors in plasma from patients with nephrotic syndrome

Data are shown for 32 protease inhibitors assayed in the Human Protease/Protease Inhibitor Array Kit (R&D Systems) and 10 patients. SRNS1-1715 (6 patients) had SRNS and were included in the MS proteomics studies. 252-5618 (3 patients) had SRNS and 2704 had SSNS. Protease inhibitors which are increased in relapse relative to remission are shown in red, those which are decreased are shown in blue. X denotes cases where the protease inhibitor quantification for either the relapse or remission plasma sample was equivalent to the negative control and a fold change could not be calculated.

As for the proteases, overall there was lack of consistent patterns in the change in protease inhibitors across all patients. However, there does not appear to be as clear an association between the geometric mean of all relapse/remission fold change ratios for all 32 protease inhibitors and the relapse/remission uPCR ratios (Figure 6.6; Spearman $r = 0$, $p > 0.99$).

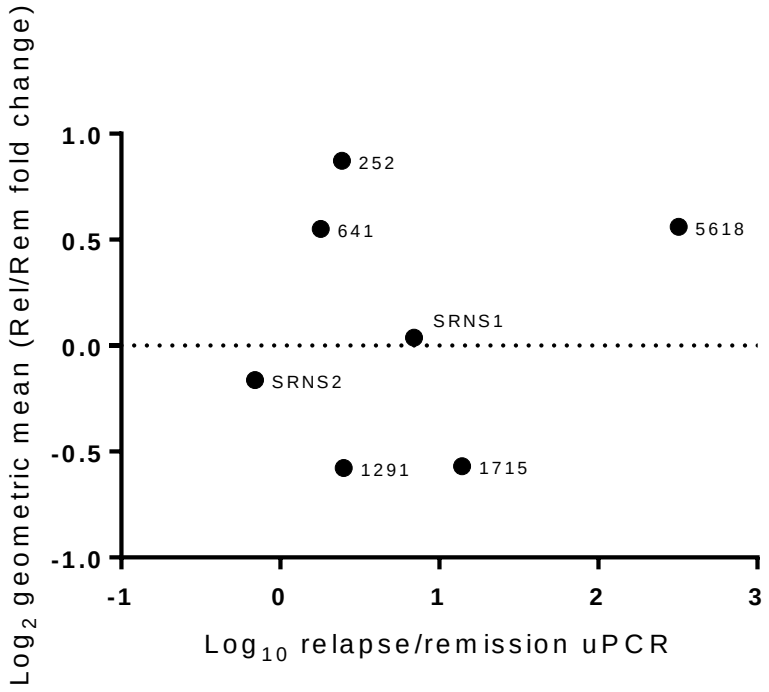


Figure 6.6: Relationship between the geometric mean of relapse/remission fold change ratio for all protease inhibitors and the relapse/remission uPCR ratio

The absence of the negative correlation seen previously for proteases, suggests that other factors may be influencing the relative quantities of protease inhibitors apart from the comparative degree of proteinuria in relapse. For example, patient 5618 had a uPCR 320 times greater in post-transplant relapse versus remission (2915 versus 9.1 mg/mmol) and despite this 22 (68.8%) of the 32 protease inhibitors were increased > 1.3-fold at the time of relapse.

A volcano plot summarising the geometric mean relapse/remission fold change and t-test p values for the 32 protease inhibitors is shown in Figure 6.7.

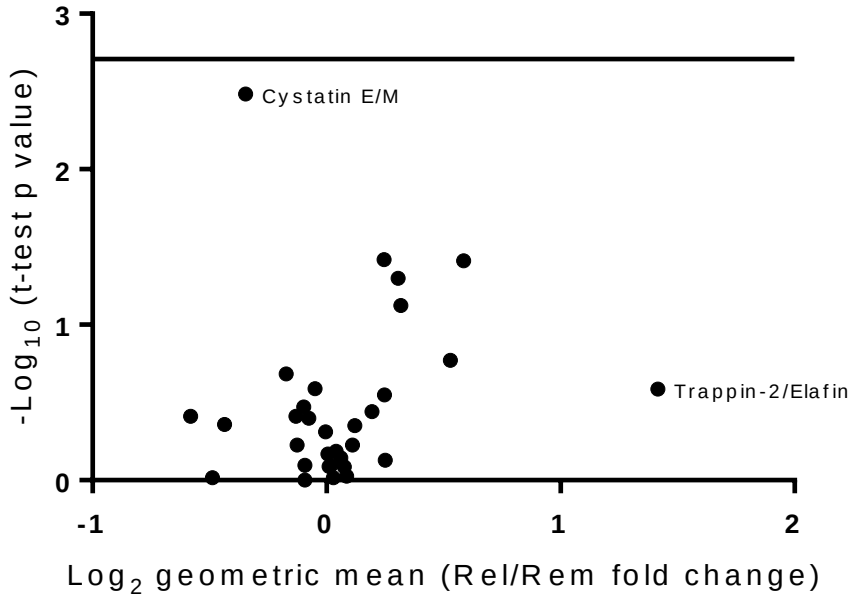


Figure 6.7: Volcano plot of protease inhibitor quantification data for relapse/remission plasma samples from patients with nephrotic syndrome

The horizontal line shows the Bonferroni-corrected significance threshold for 32 proteins with $\alpha = 0.05$

Trappin-2 appeared to be greatly increased in relapse versus remission but the geometric mean was distorted by very low quantification in the remission sample of patient 252 (relapse/remission of 227.2/0.1 = 2269.1). Exclusion of this value resulted in a geometric mean relapse/remission fold change of 1.15 among the other 8 patients for whom the ratio could be calculated. Cystatin E/M showed the most significant change (with geometric mean relapse/remission fold change of 0.79). However, no protease inhibitor reached the Bonferroni-corrected significance threshold for multiple comparisons or were significant after the BH procedure with FDR at 10%.

An analysis using fold change thresholds of > 1.3 and < 0.77 with the aim of maximising consistency across patients was performed as previously and is shown in Table 6.3.

Table 6.3: Protease inhibitors consistently increased or decreased in relapse versus remission across 10 patients with nephrotic syndrome

Target	SRNS1	SRNS2	SRNS3	560	641	1715	252	1291	5618	2704	n > 1.3	n < 0.77
Cystatin A	2.03	1.33	1.99	1.68	1.04	0.66	1.47	0.38	1.40	7.97	7	2
Lipocalin-1	2.46	1.06	2.69	1.66	1.70	0.46	3.40	0.38	3.85	1.26	6	2
Cystatin B	1.91	0.95	0.37	1.40	2.74	0.47	3.38	0.60	1.49	1.07	5	3
HAI-2	1.10	0.99	2.13	1.88	1.60	0.79	1.40	0.83	1.33	1.07	5	0
Cystatin E/M	0.90	0.82	0.65	1.00	0.75	0.65	0.72	0.65	1.17	0.71	0	6
RECK	0.64	0.49	0.47	1.14	3.04	0.44	2.97	0.68	1.07	0.87	2	5
Serpin E1/PAI-1	0.38	0.50	0.11	1.28	7.70	1.08	1.61	0.41	0.38	0.86	2	5

Red highlights fold change > 1.3 and blue < 0.77. Patient 2704 had SSNS, all others had SRNS. Proteins reaching the threshold in ≥ 5 patients are shown.

Cystatin A showed the largest and most consistent increase in relapse relative to remission but was reduced below the 0.77-fold change threshold in 2 patients. It is a cysteine protease inhibitor and studies have suggested it may act as a tumour suppressor in oesophageal cancer and inhibit epithelial to mesenchymal transition in lung cancer [368]. There are no published reports associating cystatin A with renal disease or NS.

Cystatin E/M was the most consistently decreased in relapse, below the threshold of 0.77 in 60% of patients. It is a protease inhibitor with tumour suppressor function and is most highly expressed in skin epidermis [369, 370]. It can bind and inhibit cathepsins L and V which are proteases with a function, amongst others, of converting pro-uPA (urinary plasminogen activator) to active uPA. There have been no studies directly linking cystatin E/M with renal disease in the published literature.

For the 32 protease inhibitors analysed, the median molecular weight was 39 kDa (range 11 – 106 kDa). There was no significant correlation between protease inhibitor molecular weight and $\log_2$ geometric mean of the relapse/remission fold change.

6.4 Discussion

6.4.1 Findings of this study

This study set out to identify proteins which were differentially present in plasma of patients with NS at times of relapse and remission and specifically examined proteases and protease inhibitors as these may have a direct pathogenic role in NS.

The more-targeted analysis of plasma proteases demonstrated that cathepsin E and kallikrein 5 showed the largest and most significant changes between relapse and remission (although both below the statistical significance threshold after correction of multiple comparisons). Both were decreased in relapse, but given the strong negative correlation between the overall relapse/remission ratio and degree of proteinuria (see Figure 6.3), it may be that the explanation of the change is that they are being lost in the urine. In that case, they may have no benefit as a biomarker over measurement of plasma albumin and uPCR. Similarly, the protease most increased in relapse was seen in the patients with the least relative proteinuria.

Regarding protease inhibitors, cystatin A showed the most consistent increase in relapse compared with remission and cystatin E/M showed the most consistent decrease. No previous studies have associated these with renal disease and the relevance to SRNS is currently unclear.

As discussed previously in Chapter 5, a longitudinal study with repeated samples at multiple time points would allow correlation of the plasma protease or protease inhibitor concentrations with uPCR and understanding of whether measurement can predict disease relapse or post-transplant recurrence. If changes occurred in protein levels before the onset of relapse it would also warrant further investigation to assess whether it may be a pathogenic circulating factor.

6.4.2 Comparison of potential biomarkers from literature with protease and protease inhibitor data

As discussed in Chapter 5, NGAL has been highlighted as a potential biomarker and MS showed it was increased > 1.3-fold in relapse versus remission in 7 of 8

patients tested (see Table 5.14). NGAL was also assayed in the current study in 10 patients (9 with SRNS, 1 with SSNS) using the Human Protease Inhibitor Array Kit (Section 6.3.2.2). This did not show consistent results between the subjects (Figure 6.5), with only 2 cases having relapse/remission fold change greater than 1.3 and 3 cases having less than 0.77. A direct comparison of results for NGAL using the two techniques is shown in Table 6.4. There was less consistency in the results using the protease inhibitor kit. However, the direction of change was concordant between the techniques for 5 of the patients, with discordance only for 1715.

Table 6.4: Relapse/remission ratio of NGAL in patients with SRNS assayed using mass spectrometry and human protease array kit

	SRNS1	SRNS2	SRNS3	560	641	1715
MS Rel/Rem ratio	2.60	0.85	3.00	1.43	2.38	2.03
Protease array Rel/Rem ratio	1.27	0.57	1.40	1.22	1.96	0.63

Red highlights fold change > 1.3 and blue < 0.77.
Legend: MS, mass spectrometry; Rel, relapse; Rem, remission

The literature has highlighted the protease hemopexin as a potential biomarker or circulating factor in NS [158, 173, 257], however this was not assayed by the Human Protease Array Kit, therefore, comparisons are not possible.

6.4.3 Limitations

This study used the same patient plasma samples as discussed previously in Chapter 5, therefore, shares the same limitations including the relatively small

number of patients, heterogeneity between them and differences in sample handling and processing.

6.4.4 Conclusion

Due to variability between patients, this study did not identify proteases or protease inhibitors which were significantly increased or decreased in plasma during NS relapse versus remission. However, there was some concordance between results here and MS data for NGAL, which has previously been suggested as potential biomarkers in other studies. Further analysis of this protein in a larger cohort of patients with longitudinal sampling would help to determine its value as a predictive indicator of disease.

Chapter 7 Phosphoproteomics of podocytes after SRNS plasma treatment

7.1 Introduction

As discussed in Chapter 1, several strands of evidence point to the existence of pathogenic circulating factors in non-genetic SRNS (Section 1.4.1). One such observation is the recurrence of proteinuria within hours of transplantation of a healthy donor kidney. Biopsy of the transplant, in one published example, revealed typical features of podocyte effacement [142]. The search to identify one or more of the circulating factors is ongoing. Despite this, their presence can be inferred from the effect of plasma from patients with post-transplant recurrence on *in vitro* podocyte cultures [175].

7.1.1 Conditionally immortalised human podocytes

A conditionally-immortalised human podocyte cell line was developed by Bristol Renal at the University of Bristol and originally described in 2002 [187]. Primary human podocytes were derived from a nephrectomy specimen of a 3-year-old child with hydronephrosis. There were no features of dysplasia or known primary glomerular pathology and so the podocytes were considered to be normal. Differentiated podocytes *in vitro* enter terminal growth arrest, therefore, primary podocyte cultures were transfected with a retroviral vector carrying the SV40 large T antigen gene, which promoted ongoing cell division at the permissive temperature of 33°C and terminal differentiation at 37°C. These cells were shown to express podocyte-associated proteins including podocin and nephrin. They

have been used to evaluate podocyte structural and functional responses in models of diabetes and SRNS.

As discussed in Chapter 1 (Section 1.4.4), Harris *et al.* demonstrated that VASP in podocytes was consistently phosphorylated in response to FSGS relapse plasma [188]. Phosphorylation or dephosphorylation of multiple proteins within podocytes, including nephrin, NEPH proteins, focal adhesion kinase (FAK) and synaptopodin, are involved in regulation of the actin cytoskeleton and maintenance of foot process integrity [371]. Disruption of this is central to the pathogenesis of proteinuria in SRNS.

7.1.2 Phosphoproteomics

Phosphorylation of proteins is one of the commonest post-translational modifications and is often key to regulation of their activity and function. A protein may be phosphorylated (or subsequently dephosphorylated) at multiple different amino acid residues. An ability to localise phosphorylation sites as well as quantify and compare levels of phosphoproteins in samples can give an insight into dynamic regulation of protein function and signalling pathways [372].

Chapter 5 described proteomics technologies which enabled quantification of multiple proteins in biological samples. Similar principles apply to phosphoproteomics, in the case of this study in relation to proteins extracted from podocytes. Two of the key steps include:

1. Differential labelling of all the proteins within different samples such that they can be mixed and processed in parallel for LC-MS/MS. Subsequently the quantification data can be correctly assigned to the appropriate samples.

2. Selective enrichment of the phosphopeptide fraction of the samples. The phosphopeptides represent a small proportion of all peptides after trypsinisation of a complex mixture of proteins in a cell extract. Enrichment of phosphopeptides reduces the complexity and enhances the likelihood of accurate peptide identification and quantification by LC-MS/MS [373].

Differential labelling of proteins may be achieved in a variety of ways. In this study, 10-plex TMT labelling, as described in Chapter 5 (Figure 5.3), was used since this provides the benefit of parallel processing and analysis of 4 relapse/remission pairs of samples together with two controls.

Several methods of phosphoprotein enrichment have been described including immobilised metal affinity chromatography and immunoaffinity purification [374]. In this study, TiO_2 chromatography was employed which is based on the very high affinity of the metal oxide for phosphoproteins in an acid environment. After separation of non-bound peptides, the phosphopeptides are eluted using an alkaline buffer. The whole process has a high specificity for phosphorylated versus non-phosphorylated peptides and is generally very tolerant to the presence of salts and detergents in the protein extraction mixture [375].

7.1.3 Phosphoproteomics of renal cells

Proteomic analysis of the renal filtration barrier has provided an insight into signalling pathways which can be affected in proteinuric disease [376]. The potential pathogenic role of disruption of intracellular and extracellular podocyte proteases has recently been reviewed [377]. Several studies have examined the

phosphoproteome of glomeruli and podocytes in particular. The most comprehensive examined the phosphoproteome of mouse glomeruli and identified 2449 phosphorylated proteins with 146 phosphorylation sites on proteins expressed at high levels in podocytes [378]. Synaptopodin was one with the highest number of phosphorylation sites but other phosphorylated proteins included podocin, nephrin and CD2AP. The same research group also examined the phosphoproteome of cultured mouse podocytes and identified increased phosphorylation of actin-filament associated proteins in fully-differentiated cells [379].

The aim of this study was to compare levels of phosphoproteins in podocytes shortly after treatment with plasma from SRNS patients at times of disease relapse and remission. The intention was both to highlight potentially novel pathogenic signalling pathways and identify podocyte phosphoproteins which could act as biomarkers to differentiate relapse and remission plasma.

7.2 Methods

7.2.1 Patients

The same patients were included in this study as previously described in Chapter 5: C1, SRNS1, SRNS2, SRNS3 and 419. In the case of patient 419, REL-2 was used as the relapse sample as this was associated with more consistent VASP phosphorylation activity in previous experiments (D. Henson, personal communication).

7.2.2 Tissue culture of conditionally-immortalised human podocytes

Tissue culture was performed using podocytes as described previously in Chapter 5 (Section 5.2.4).

7.2.3 Treatment of podocytes with patient plasma

On the day of treatment, standard medium was removed and replaced with RMPI 1640 lacking FBS and ITS. Podocytes were incubated for 2 hours in serum-free (SF) medium before treatment started. Plasma was diluted in SF RPMI 1640 to a final concentration of 10% in a total volume of 4ml. Paired relapse and remission samples were used from the patients with SRNS. After applying the treatment, podocytes were returned to 37°C for 30 minutes.

7.2.4 Protein extraction and preparation

Protein extraction followed the same method as described in Chapter 5 (Section 5.2.6). After centrifuging, the supernatant was transferred to fresh Eppendorfs and the protein concentration determined using the BCA assay. A total of 100 μg protein was sent to the University of Bristol Proteomics Facility for total and phosphoproteomic analysis. Remaining supernatant was stored at -80°C for future validation experiments.

7.2.5 Quantitative total and phosphoproteomics

Quantitative total and phosphoproteomics was performed at the University of Bristol Proteomics Facility as previously described [319]. Briefly, 10 samples were digested with trypsin overnight, labelled with TMT 10-plex reagents and pooled together. For total proteomics, the pooled samples were fractionated by

high-pH reversed-phase chromatography. The fractions were analysed by nano-LC-MS/MS using an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). For phospho-proteomics, samples were processed using TiO_2-based phosphopeptide enrichment (Pierce) followed by nano-LC-MS/MS. Data files were processed and analysed with Proteome Discoverer software (v1.4, Thermo Scientific) and searched against the Uniprot Human database (134169 sequences) using the SEQUEST algorithm. The search was performed with full tryptic digestion allowing a maximum of one missed cleavage. The FDR was set at 1% for all peptide data using the reverse database search option

7.2.6 Phosphoproteomics data analysis

Quantification data for peptides from phospho-enriched samples were analysed in Proteome Discoverer. The peptide FDR was set at 1%. The relative fold change was calculated as the ratio of quantity in the "relapse" sample to quantity in "remission" for the same patient using the Proteome Discoverer Ratio Reporting function. The ratios for each sample to SF control and to C1 control were also determined. The data were then exported to Microsoft Excel for further analysis.

Only peptides assigned to a single protein group and with complete quantification data were included. Each peptide assigned to a single protein was also associated with a UniProt accession number. However, in many cases several proteins each with their own accession number, were a member of a single protein group. Proteome Discoverer assigned the protein group with one accession number belonging to the constituent protein with the greatest coverage (that is, the one identified with the highest confidence based on the MS data). In order to minimise mismatches when comparing between datasets and using

bioinformatics tools, all accession numbers from the protein group together with the gene name associated with each peptide were used.

In order to identify biological pathways of potential interest, peptides in the phospho-enriched samples which changed by > 2 or < 0.5 in podocytes treated with relapse versus remission plasma from a single patient were inputted into STRING to analyse potential interacting proteins [380]. The following settings were adjusted to favour a more stringent analysis:

- Textmining was deselected as one of the active interaction sources
- The minimum required interaction score was set at the highest confidence (0.900)

When displaying the protein interaction diagram, disconnected nodes in the network were hidden. The main biological functions of altered proteins were summarised using KEGG pathway analysis within STRING [381, 382]. The peptide data for each patient relapse/remission pair were inputted separately and the affected pathways compared for similarity.

In order to identify differences in phosphorylation of proteins between relapse and remission plasma treatments with a view to more detailed *in vitro* validation, the list of peptides was filtered to those with phospho modifications and those with fold changes > 2 or < 0.5 most consistent across four patients. The location of the peptide phospho modification in the parent protein was determined using PhosphoSitePlus ® (freely available at www.phosphosite.org) [383]. In the initial analysis of the four patients (SRNS1, SRNS2, SRNS3 and 419) only the peptide data from the phospho-enriched samples were used and it was assumed that the associated total proteins would not change during the 30-minute treatment

with relapse or remission plasma. These findings were used to direct further *in vitro* studies as discussed below.

7.2.7 Western blotting for phosphoproteins

The antibodies used for Western blotting are shown in Table 7.1.

Table 7.1: Antibodies used in Western blotting

Target	MW (kDa)	Product code	Manufacturer	Species	Dilution
pBAD (Ser112)	23	9291	CST	Rabbit	1:1000
BAD	23	9292	CST	Rabbit	1:1000
Palladin	95 & 140	10853-1-AP	Proteintech, Rosemont, IL, USA	Rabbit	1:2000
Phosphoserine	-	Ab9332	Abcam	Rabbit	3 µg/mL
GAPDH	36	MAB374	Merck Millipore	Mouse	1:10000

7.2.8 Immunoprecipitation

Table 7.2: Solutions used for immunoprecipitation

IP extraction buffer	150 mmol/L NaCl
	20 mmol/L Tris base pH 7.5
	10% (v/v) Glycerol
	1% (v/v) Triton® X-100
Supplements added to IP buffer just prior to use	1% (v/v) Protease inhibitor cocktail (Sigma-Aldrich; #P8340)
	1% (v/v) Phosphatase inhibitor cocktail 2 (Sigma-Aldrich, #P5726)
	1% (v/v) Phosphatase inhibitor cocktail 3 (Sigma-Aldrich; #P0044)
	400 µmol/L Phenylmethane sulfonyl fluoride (PMSF)
TNE buffer	50 mmol/L Tris HCl pH 7.4
	150 mmol/L NaCl
	0.5 mmol/L EDTA

Podocytes were cultured, as previously described, in T175 flasks to provide sufficient protein for further analysis. They were treated with plasma for 30 minutes and proteins extracted using IP extraction buffer with protease and phosphatase inhibitor supplements (Table 7.2) before centrifuging and storing the supernatant at -80°C for future use.

Protein A/G Plus agarose beads (10% v/v; Santa-Cruz, #sc-2003) were resuspended in TNE buffer (Table 7.2). Anti-palladin IgG (5 μg) and normal rabbit IgG (5 μg) were added to separate aliquots of 200 μL resuspended agarose beads in Eppendorf tubes. These were placed on a rotator overnight at 4°C.

Podocyte lysates were defrosted for pre-clearing. 10% BSA in TNE was added to give a final BSA concentration of 1% followed by 20 μL agarose beads. Tubes were rotated at 25 rpm for 1 hour at 4°C before being centrifuged at 1000 × g for 1 minute at 4°C. The supernatants were transferred to fresh tubes and kept on ice.

The Eppendorf tubes containing agarose beads with anti-palladin and rabbit IgG were also centrifuged at 1000 × g for 1 minute at 4°C before discarding the supernatant. The beads were washed twice by resuspending in 200 μL TNE buffer, centrifuging and discarding the supernatant. Half the volume of pre-cleared cell lysate was added to palladin IgG-beads and the rest to normal rabbit IgG-beads and the volumes made up to 500 μL. The mixtures were rotated at 25 rpm for 3 hours at 4°C and then centrifuged at 1 × g for 1 minute. The beads were washed four times by resuspending in 500 μL and then centrifuging at 1 × g for 1 minute.

Palladin and rabbit IgG immunoprecipitates and whole cell lysate were prepared for SDS-PAGE by adding 2 × sample buffer with 2-mercaptoethanol and

boiling for 10 minutes at 95°C. The samples were centrifuged at 1000 × g for 1 minute to remove bead fragments before proceeding to load on a 10% polyacrylamide gel for SDS-PAGE and Western blotting as previously described.

7.3 Results

7.3.1 Patient characteristics

The demographic details and data relating to plasma samples were as detailed previously in Chapter 5.

7.3.2 Phosphoproteomic analysis of podocytes treated with relapse and remission plasma from patients with SRNS

Cell culture extracts from podocytes treated with SF media and plasma from C1, SRNS1, SRNS2, SRNS3 and 419 were analysed with LC-MS/MS. Quantification data relating to peptides in the phospho-enriched fraction were extracted and the quantification of each plasma sample relative to SF media were examined. Of 4217 peptides identified, 5 were not matched to a protein, 245 were assigned to more than one protein group and 24 had incomplete quantification data leaving 3943 peptides with unique protein matches for analysis. Peptides that were increased or decreased in plasma-treated podocytes versus SF media were identified and the total numbers are shown in Table 7.3.

Table 7.3: Number of peptides in phospho-enriched podocyte extracts reaching fold-change thresholds after treatment with plasma versus serum-free media

Sample		C1	SRNS 1 Rel	SRNS 1 Rem	SRNS 2 Rel	SRNS 2 Rem	SRNS 3 Rel	SRNS 3 Rem	419 Rel	419 Rem
Plasma/SF fold-change threshold	< 0.5	50	84	71	156	91	82	180	40	48
	> 2	297	364	500	292	346	343	190	153	244
Total		347	448	571	448	437	425	370	193	292

Total number of peptides with quantification data available = 3943
Legend: Rel, relapse; Rem, remission, SF, serum-free media

Treatment of podocytes with patient plasma was associated with changes in peptide quantities compared with treatment with SF media. Between 4% (491 relapse) and 14% (SRNS1 remission) of quantified peptides had a fold change > 2. Of note, a similar number of peptides were affected by treatment with plasma from the control subject C1 who did not have SRNS. In order to understand the possible effects of plasma on the podocytes, the interactions between proteins identified from the peptides were investigated using STRING.

Peptides that were increased or decreased in response to treatment with relapse versus remission plasma were identified and the total numbers used for pathway analysis are shown in Table 7.4.

Table 7.4: Number of peptides in phospho-enriched podocyte extracts reaching relapse/remission fold-change thresholds and used for STRING pathway analysis

Patient		SRNS1	SRNS2	SRNS3	419
Relapse/remission fold-change threshold	< 0.5	130	116	50	94
	> 2	40	52	311	59
Total inputted for STRING analysis		170	168	361	153
Total recognised and used in STRING analysis		148	158	290	139

Total number of peptides with quantification data available = 3943

As described in the Methods, the STRING search used all accession numbers from each protein group corresponding to the identified peptides. However, in some instances several peptides matched to the same protein hence the number used in analysis is fewer that the number inputted due to removal of these duplicates. It was evident that treatment with paired plasma from SRNS3 was associated with a greater number of peptide changes and in an opposite direction (more peptides increased in relapse) compared with the other patients.

Figure 7.1 to Figure 7.4 show the networks of protein interactions predicted based on peptides increased or decreased by treatment with relapse versus remission patient plasma. In each case the number of interactions (edges) was significantly greater than expected by chance based on a set of proteins of similar size randomly selected from the genome.

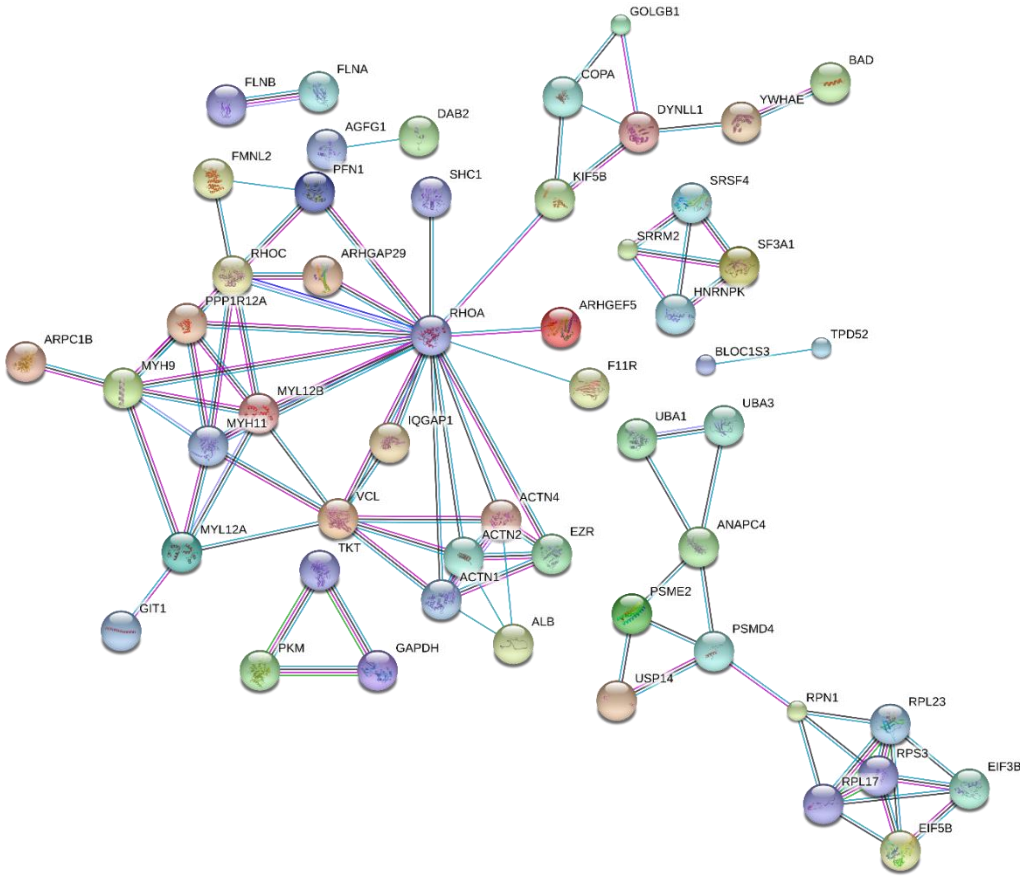


Figure 7.1: Protein interaction network based on peptide quantification ratios > 2 or < 0.5 in podocytes treated with relapse versus remission plasma from patient SRNS1

Legend: Each circle represents a protein and is annotated with the gene name. Only proteins with at least one connection are shown.
Diagram created using STRING available at https://string-db.org [332]

Number of nodes (proteins): 146
Number of edges (connections): 93
Expected number of edges: 51
Protein-protein interaction (PPI) enrichment p value: 7.11×10^{-8}

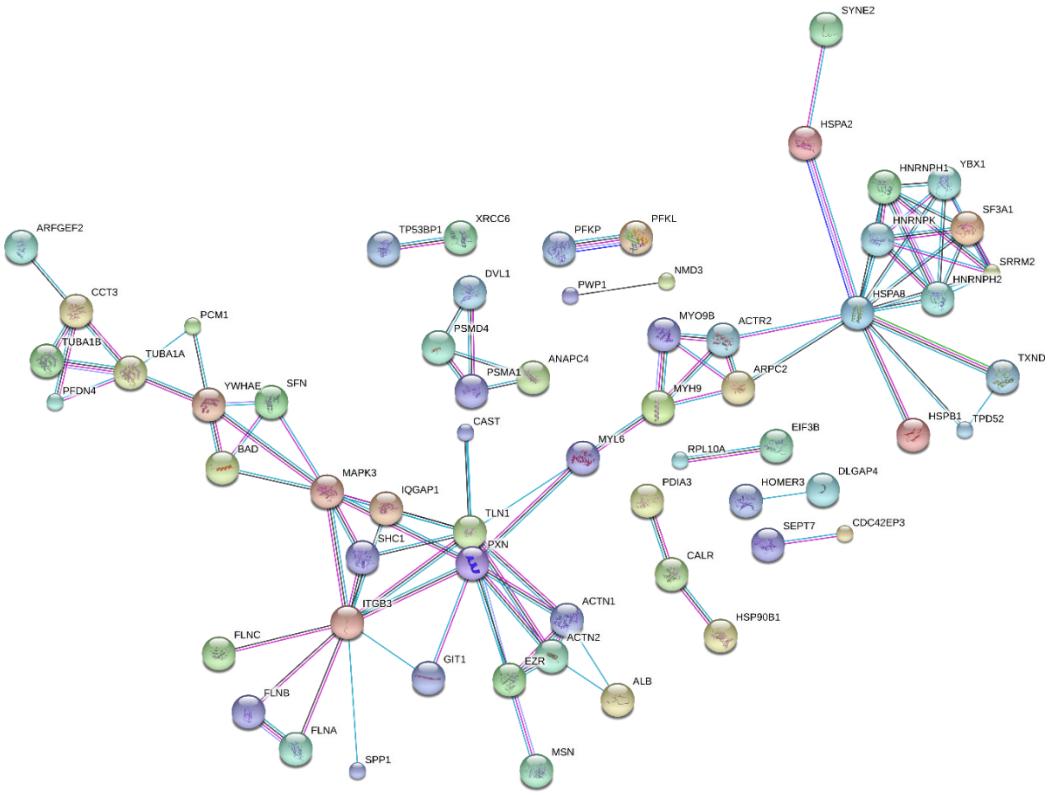


Figure 7.2: Protein interaction network based on peptide quantification ratios > 2 or < 0.5 in podocytes treated with relapse versus remission plasma from patient SRNS2

Legend: See Figure 7.1

Number of nodes (proteins): 158
Number of edges (connections): 99
Expected number of edges: 45
Protein-protein interaction (PPI) enrichment p value: 2.18×10^{-12}

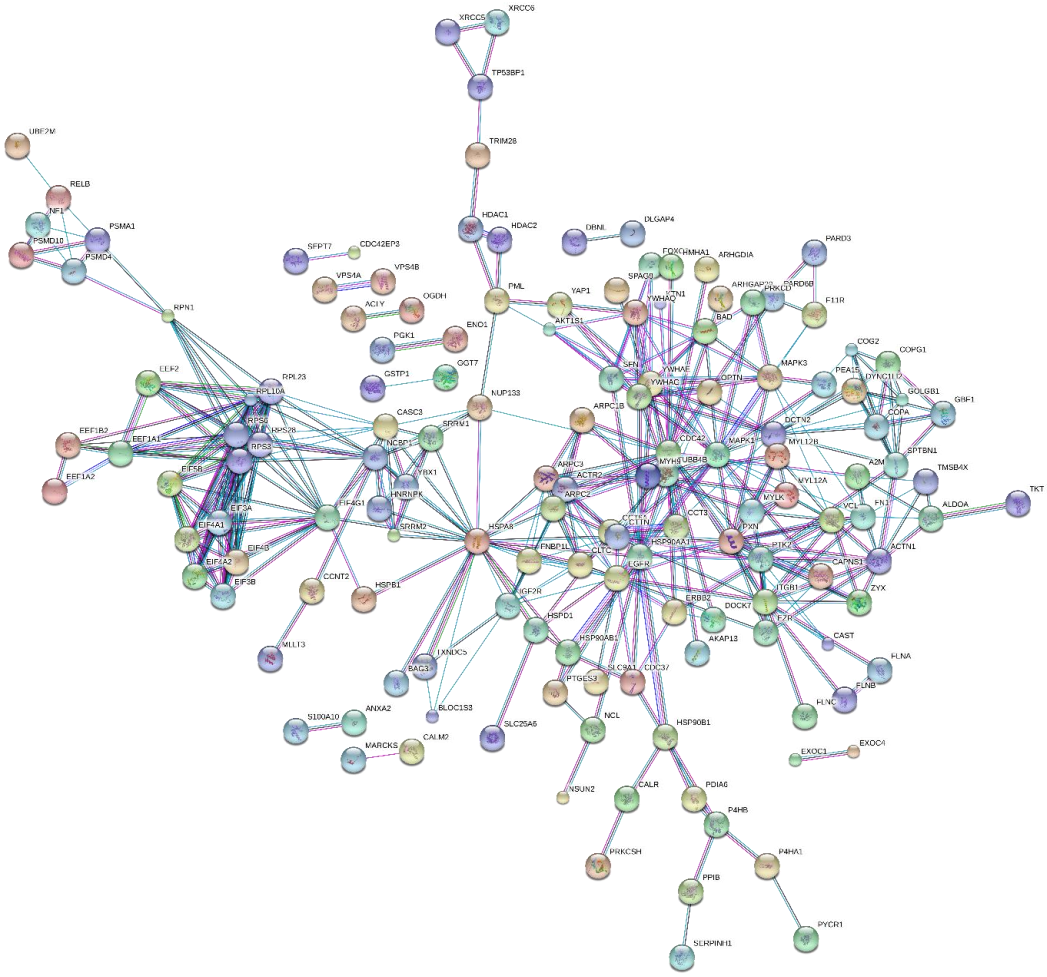


Figure 7.3: Protein interaction network based on peptide quantification ratios > 2 or < 0.5 in podocytes treated with relapse versus remission plasma from patient SRNS3

Legend: See Figure 7.1

Number of nodes (proteins): 290
Number of edges (connections): 432
Expected number of edges: 215
Protein-protein interaction (PPI) enrichment p value: 0

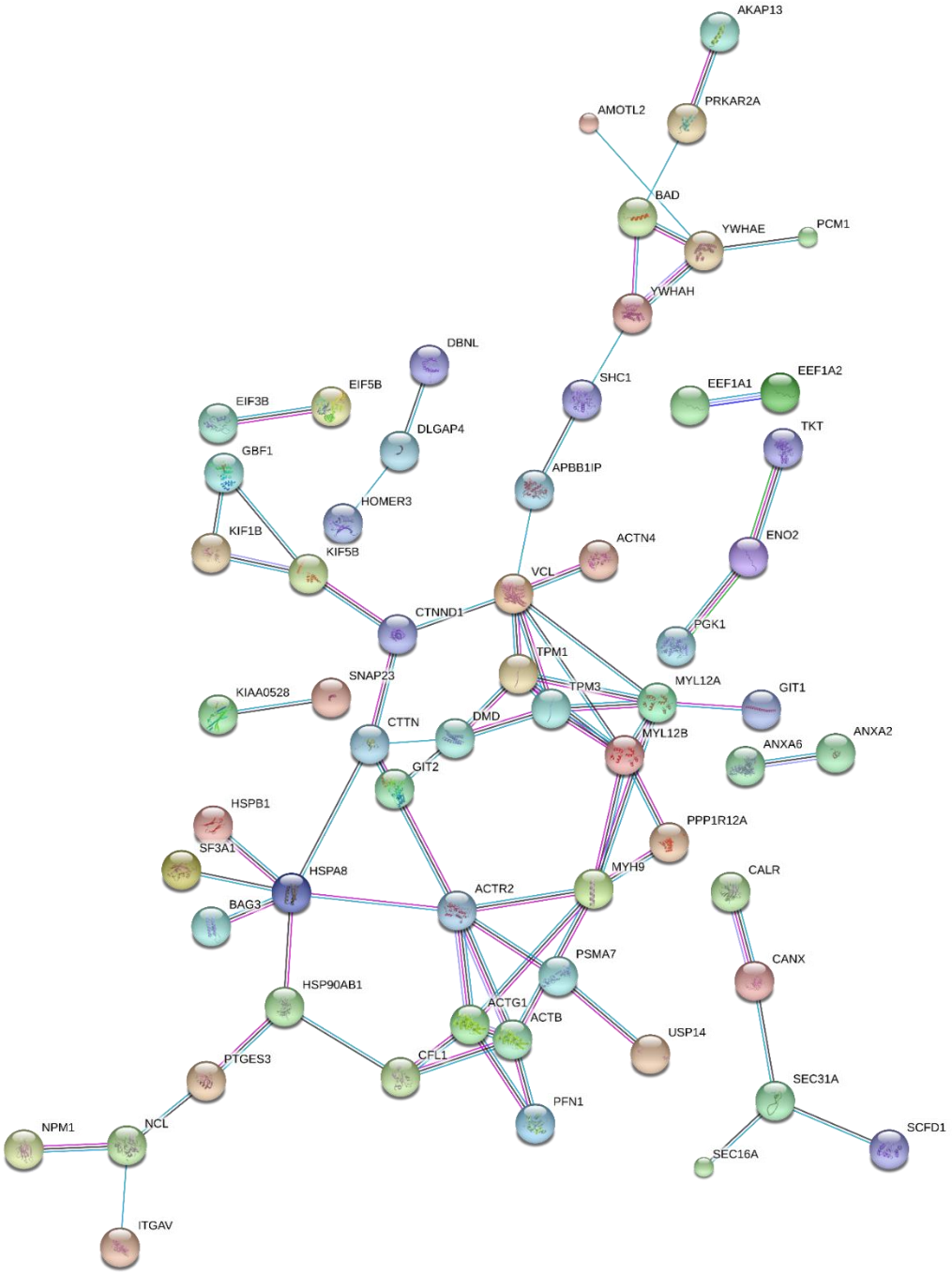


Figure 7.4: Protein interaction network based on peptide quantification ratios > 2 or < 0.5 in podocytes treated with relapse versus remission plasma from patient 419

Legend: See Figure 7.1

Number of nodes (proteins): 139
Number of edges (connections): 73
Expected number of edges: 28
Protein-protein interaction (PPI) enrichment p value: 8.66×10^{-13}

KEGG pathway analyses for the four relapse/remission plasma samples are shown in Table 7.5.

Table 7.5: Top 5 KEGG pathways most likely affected differentially by treatment with relapse or remission plasma from patients with SRNS

Patient	Pathway description	Number of proteins	FDR
SRNS1	Regulation of actin cytoskeleton	15	7.30×10^{-9}
	Focal adhesion	14	2.94×10^{-8}
	Tight junction	11	1.75×10^{-7}
	Leukocyte transendothelial migration	9	8.55×10^{-6}
	Adherens junction	6	0.00044
SRNS2	Focal adhesion	12	1.15×10^{-5}
	Regulation of actin cytoskeleton	12	1.15×10^{-5}
	Salmonella infection	7	0.000296
	Protein processing in endoplasmic reticulum	9	0.000345
	Viral carcinogenesis	8	0.00481
SRNS3	Regulation of actin cytoskeleton	21	6.48×10^{-10}
	Focal adhesion	19	1.28×10^{-8}
	Bacterial invasion of epithelial cells	12	4.27×10^{-8}
	Pathogenic Escherichia coli infection	10	1.88×10^{-7}
	Proteoglycans in cancer	17	8.85×10^{-7}
419	Regulation of actin cytoskeleton	12	5.49×10^{-6}
	Thyroid hormone signalling pathway	9	1.30×10^{-5}
	Focal adhesion	11	1.32×10^{-5}
	Leukocyte transendothelial migration	7	0.000699
	Bacterial invasion of epithelial cells	6	0.000699

Legend: FDR, false discovery rate

As evident from the descriptions, proteins in the pathways “regulation of actin cytoskeleton”, “focal adhesion” and “adherens junction” all have a role in

cellular motility and cell-cell interactions. The "salmonella infection" pathway also includes proteins which induce the rearrangement of the actin cytoskeleton and result in membrane ruffles. In at least three of the top five protein interaction pathways for all patients a common theme was cell motility, focal adhesions and regulation of the actin cytoskeleton.

In order to identify individual phosphoproteins for further analysis and *in vitro* validation of potential target pathways, the MS quantification data for peptides with phospho modifications from the phospho-enriched samples were examined. A fold-change threshold of > 2 was used to identify phosphopeptides increased by treatment with relapse versus remission plasma consistently across the four patients. Three peptides with the greatest fold change and consistency are shown in Table 7.6. The fold change at the level of total proteins is shown in Table 7.7.

Table 7.6: Phosphopeptides with relapse/remission fold change > 2 across four patients with SRNS

Protein names	Amino acid sequence	Modifications	SRNS1	SRNS2	SRNS3	419
Palladin, cytoskeletal associated protein	iAsDEEIQGTk DAVIQDLER	N-Term(TMT6plex); S3(Phospho); K11(TMT6plex)	11.14	27.02	11.31	0.76
cDNA FLJ61193, highly similar to Bcl2 antagonist of cell death	hSsYPAGTED DEGmGEEPSP FR	N-Term(TMT6plex); S3(Phospho); M14(Oxidation)	4.06	2.18	2.28	0.22
DLG-associated protein 4	qNsATESADSI EIYVPEAQTR	N-Term(TMT6plex); S3(Phospho)	0.85	2.60	2.63	2.43

Red highlights fold change > 2 and blue < 0.5

Table 7.7: Relapse/remission fold change at level of total proteins corresponding to identified phosphopeptides

Protein names	SRNS1	SRNS2	SRNS3	419
Palladin, cytoskeletal associated protein	1.01	0.94	1.04	0.96
cDNA FLJ61193, highly similar to Bcl2 antagonist of cell death	0.91	0.93	1.03	0.93
DLG-associated protein 4	0.95	0.84	1.13	1.08

The three phosphopeptides identified were increased in podocytes treated with relapse plasma more than two-fold compared with the treatment with remission plasma in three of the four patients. The difference in the level of phosphopeptides was not due to change in the amount of total protein which was relatively similar between the two treatments.

The size and known functions of the selected proteins are detailed in Table 7.8.

Table 7.8: Biological characteristics of proteins identified from most consistently altered phosphopeptides

Protein name	Gene	MW (kDa)	Function
Palladin	*PALLD*	151	Cytoskeletal protein required for organising actin cytoskeleton, component of actin-containing microfilaments, role in control of cell shape, adhesion and contraction
Bcl-2 associated agonist of cell death (BAD)	*BAD*	18	Positive regulator of apoptosis via heterodimers with Bcl-xL and Bcl-2 and reversing their death repressor activity. Activity regulated by phosphorylation involving protein kinases AKT and MAP kinase and protein phosphatase calcineurin
DLG -associated protein 4	*DLGAP4*	108	Membrane-associated guanylate kinase at post-synaptic density in neurones, can interact with potassium channels and receptors, may act as an adaptor protein linking ion channels to the subsynaptic cytoskeleton

A literature review was conduction to understand whether the proteins identified from the peptides may be of functional significance in the podocyte response to SRNS plasma. No studies linking DLG-associated protein 4 with podocytes were identified.

Research using cultured podocytes exposed to puromycin aminonucleoside showed they underwent apoptosis which appeared to be related to increased BAD mRNA and protein expression [384]. Treatment with dexamethasone in this setting decreased BAD mRNA and protein levels together with reducing apoptosis. A different study in patients with IgA nephropathy found that podocyte Bcl-2 was upregulated in renal biopsies from early-stage disease and downregulated in late-stage disease [385].

Palladin has been shown to co-localise with VASP at focal adhesions [386]. It is expressed in podocytes, co-localised with F-actin and was found in ring-like actin structures and ruffles [387]. Palladin mRNA levels were significantly decreased in glomerular tissue from biopsies of patients with FSGS compared with controls. This study did not examine phosphorylation of palladin in podocytes, however other research using different *in vitro* epithelial cell cultures, including HEK-293T and human breast cell lines, showed that palladin is phosphorylated at serine 77 and 197 in response to the growth factor extracellular signal-related kinase (ERK) [388]. Using immunofluorescence with cell lines expressing wild-type or mutant palladin, the authors suggested that ERK phosphorylation of palladin had an anti-migratory effect.

Based on the published research, it was decided to validate the proteomics findings of BAD and palladin phosphorylation with further *in vitro* experiments. The localisation of the S3 phospho modification in the BAD peptide was

determined using PhosphoSitePlus® as serine 75. Phosphorylation of BAD at this site has been shown to promote binding to 14-3-3 proteins which prevents interaction of BAD with Bcl-2 and Bcl-xL and, therefore, has an anti-apoptotic effect [389]. An antibody targeting phospho-BAD (Ser75) was commercially available and used in Western blotting experiments.

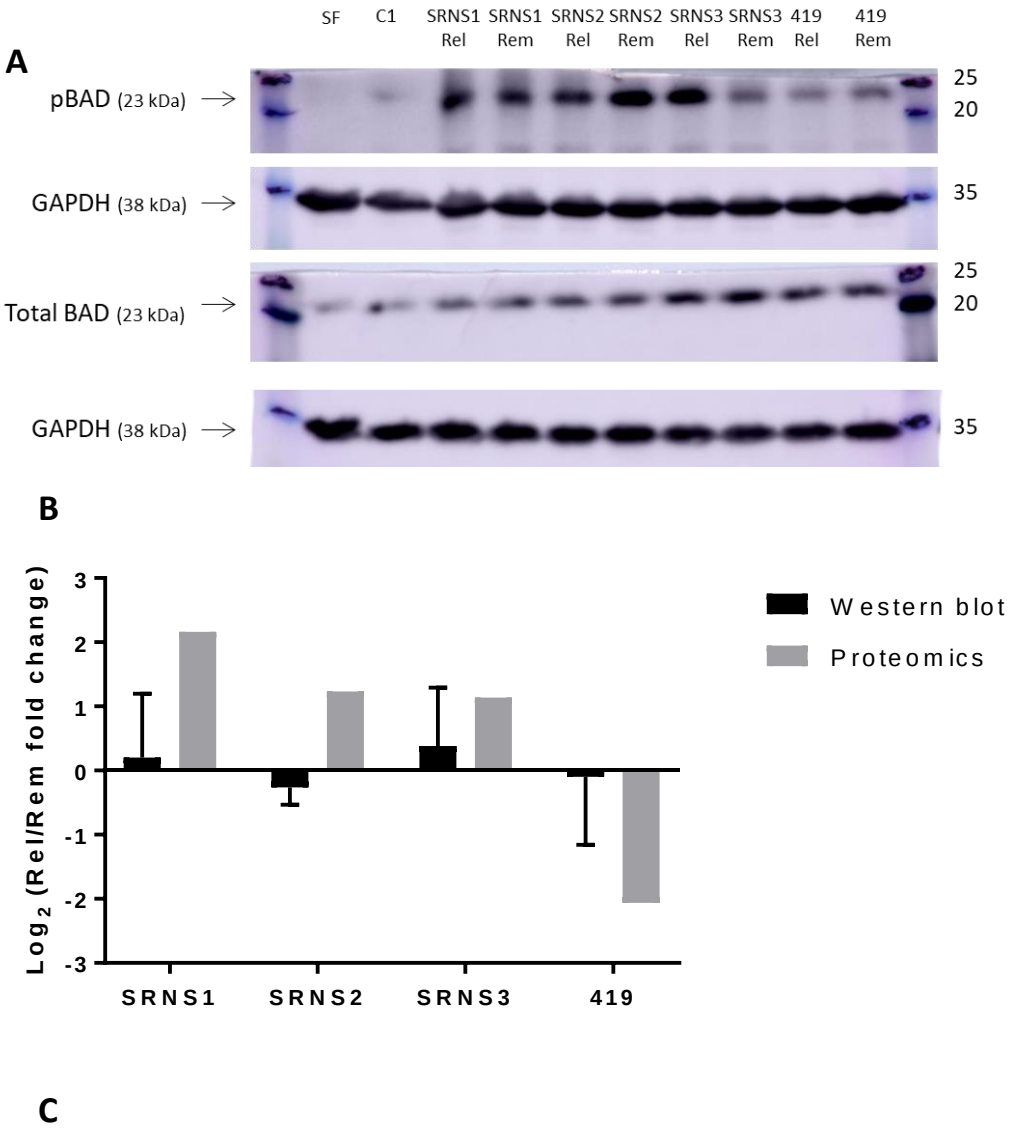
The localisation of the S3 phospho modification in the palladin peptide was determined as serine 893. Other than MS, no confirmatory or functional studies relating to phospho (Ser893)-palladin exist. A commercially-available antibody targeting this for use in Western blotting was not available, therefore a different approach to validation was planned, namely immunoprecipitation to pull down total palladin followed by blotting and detection using an anti-phosphoserine antibody.

7.3.3 Validation of phospho-BAD findings using Western blotting

Proteins extracted from podocytes after treatment with control and patient plasma were separated by SDS-PAGE and detected by Western blotting and results shown in Figure 7.5.

There was minimal BAD (Ser75) phosphorylation in podocytes in SF media. There was evidence of phosphorylation after 30 minutes' treatment with all of the plasma samples from patients with SRNS and to a lesser extent with control plasma. Although MS proteomics had suggested that the phosphopeptide was increased > 1.3-fold (> 0.38 after $\log_2$ transformation, Figure 7.5B) after treatment with relapse plasma from three patients, these findings were not confirmed by Western blotting. Based on Western blotting quantification, the relapse/remission fold change of phospho-BAD normalised to total BAD was not

significantly different from 1 for each of the patients (one-sample t test on log-transformed data, null hypothesis log-ratio = 0).



Patient	SRNS1	SRNS2	SRNS3	419
Western blotting Rel/Rem fold change (geometric mean)	1.15	0.83	1.30	0.93
Proteomics Rel/Rem fold change	4.47	2.35	2.20	0.24

Figure 7.5: Western blotting and proteomic quantification of phospho-BAD in extracts from podocytes treated with paired relapse/remission plasma samples

A: Western blot of podocyte extracts after exposure to serum-free media (SF), control (C1) and paired relapse and remission plasma samples. **B**: Log_2 transformed relapse/remission (normalised phospho-BAD/total BAD) fold change quantification from densitometry of blots (n = 4) and MS proteomics. **C**: Relapse/remission (normalised phospho-BAD/total BAD) fold change ratios determined from Western blotting and MS.

7.3.4 Validation of palladin findings using immunoprecipitation

Prior to IP experiments, Western blotting was used to confirm that total palladin could be detected in podocyte extracts and did not appear to change between treatments with relapse and remission plasma (Figure 7.6).

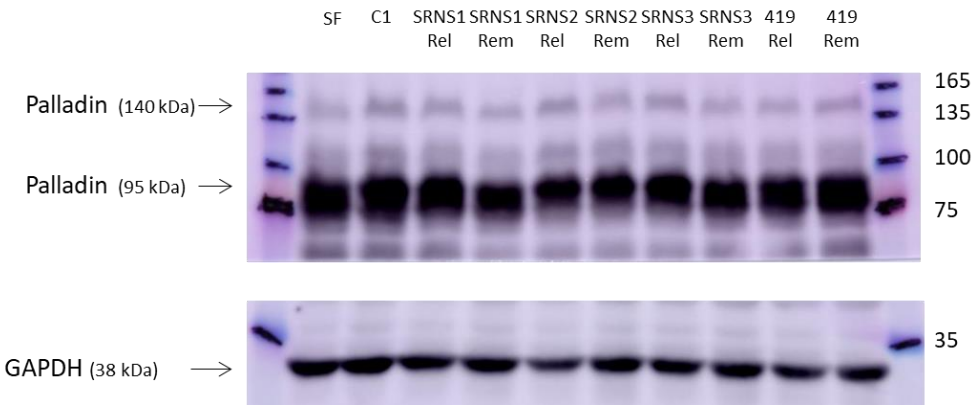


Figure 7.6: Western blotting of total palladin in extracts from podocytes treated with paired relapse/remission plasma samples

Product literature accompanying the palladin polyclonal antibody (Proteintech, #10853-1-AP) confirmed that bands were expected at 95 kDa and 140 kDa but other isoforms at 200 kDa and 65 kDa are also known to exist.

Figure 7.7 shows results from immunoprecipitation using anti-palladin antibody with lysates from podocytes treated with SF media and plasma from SRNS3 at time of relapse.

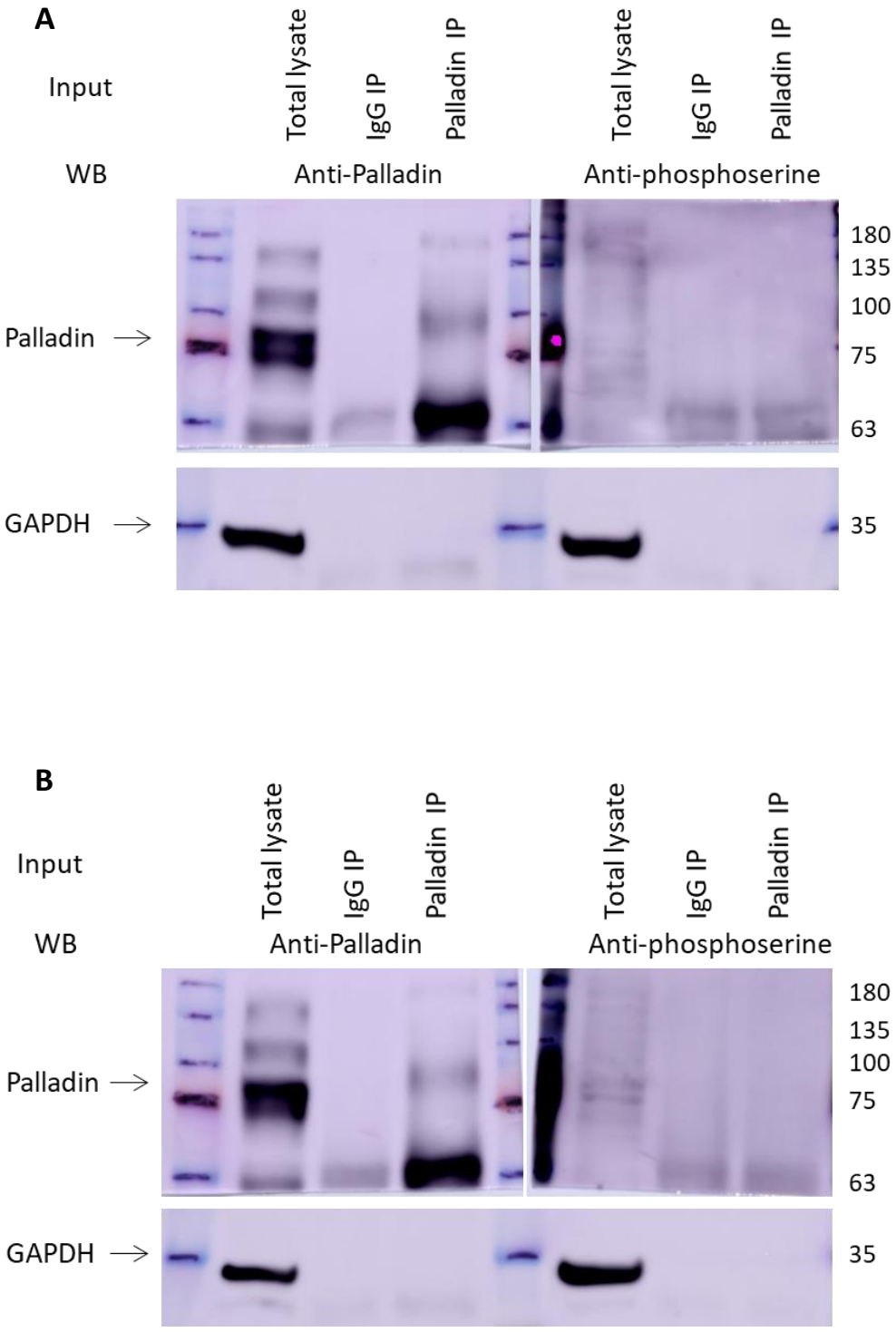


Figure 7.7: Immunoprecipitation of podocyte extracts with anti-palladin antibody and Western blotting for palladin and total phosphoserine

A: Podocytes treated with serum-free media. **B**: Podocytes treated with plasma from patient SRNS3 at time of relapse.

Total lysate from podocytes in SF media had a major band at and above 75 kDa after blotting for palladin, however following palladin IP the predominant band was just above 65 kDa (Figure 7.7A). Blotting for total phosphoserine after palladin IP detected a band also just above 65 kDa but this was similar in intensity to a band at the same position after IP with normal rabbit IgG control antibody, suggesting that this may be non-specific. When podocytes were treated with plasma from patient SRNS3 in relapse, it was expected that phospho-palladin levels would be increased based on the previous MS results. However, there was a very similar pattern to SF media (Figure 7.7B).

There appeared to be little difference in the blots using anti-phosphoserine after treatment with SF or SRNS3's plasma either in total lysate or with palladin IP. Western blotting for phosphoserine in whole cell lysate from podocytes after treatment with control, relapse and remission plasma samples showed a consistent pattern of multiple bands (Figure 7.8). There was a prominent band at 75 kDa which did not vary between relapse and remission plasma treatments. It was in a similar position as the major band when blotting for palladin but probably represented non-specific binding.

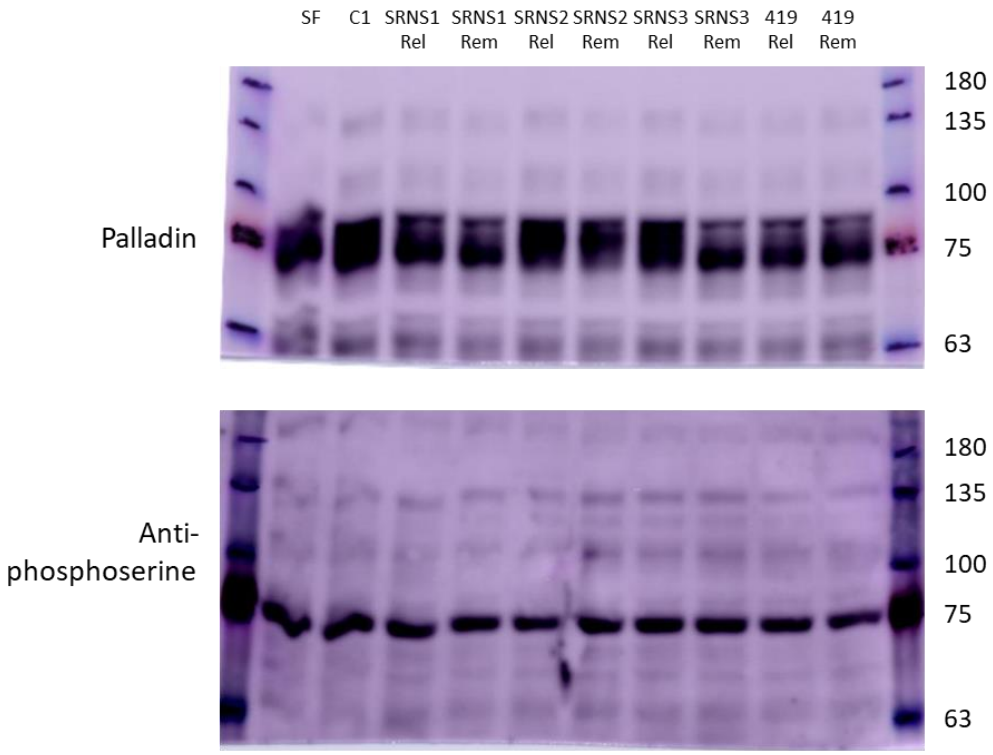


Figure 7.8: Western blotting of total palladin and anti-phosphoserine in extracts from podocytes treated with paired relapse/remission plasma samples

7.4 Discussion

7.4.1 Findings of this study and comparison with the literature

This study focused on changes in the podocyte phosphoproteome at 30 minutes after exposure to plasma from patients with SRNS. It directly compared the effects of plasma taken at the time of relapse, when circulating factor activity would be expected to be greatest, with plasma at the time of remission. For the 4 patients, the phosphoproteins with the greatest differences between relapse and remission plasma treatments were consistently within the protein interaction pathways involved in focal adhesion and regulation of the actin cytoskeleton. This supports the current understanding of early changes in actin dynamics and

podocyte motility resulting in proteinuria rapidly after transplant in patients considered to have circulating factor disease [390, 391].

After analysis of the effects of plasma treatment at the pathway level, the study sought to focus on individual phosphoproteins showing the largest difference between relapse and remission plasma treatments. Phosphopeptides derived from palladin and BAD both showed > 2-fold increase in podocytes treated with relapse plasma compared with remission from 3 of the 4 patients. Total protein quantification was almost the same with the two treatments suggesting the difference was due to increased phosphorylation rather than changes in the absolute amount of protein. Paired plasma from patient 419 did not show the same consistency in the relapse/remission fold change of phosphoprotein. The "relapse" plasma sample taken from this patient was at a time when the treating clinical team judged them to be in relapse (419 REL-2 sample), however the uPCR was 81 mg/mmol and plasma albumin was 37 g/L. An alternative explanation may be that the circulating factors in 419 were different from the other patients, and so activated distinct intracellular pathways in podocytes. Although these may be reasons for the difference from other patients, 419 did show more consistent relapse/remission fold change for other phosphoproteins.

Attempts were made to validate the phosphoproteomic findings for both BAD and palladin but neither could be confirmed using the techniques in this study. Western blotting for phospho (Ser75)-BAD did not show a biologically or statistically significant increase in relapse versus remission plasma-treated podocytes.

The phosphoproteomic data for palladin, with relapse/remission ratios > 10 for patients SRNS1, SRNS2 and SRNS3, was particularly striking. It was considered whether these data could be outliers however the consistency across 3 of the 4 patients warranted further investigation. An initial literature review and knowledge that palladin is expressed in podocytes with a role in organisation of the actin cytoskeleton provided further justification for ongoing study. The absence of a commercially-available anti-phospho (Ser893)-palladin antibody made direct validation of the phosphoproteomic findings more challenging. The use of palladin IP followed by Western blotting for phosphoserine was hampered by non-specific binding and would not have been able to confirm phosphorylation specifically at Ser893.

Since completion of these laboratory experiments, further research has been undertaken by others examining in detail the role of palladin in podocytes [392]. Artelt *et al.* (2018) conducted studies using knock-down of palladin in cultured podocytes and mice with podocyte-specific knock-out of palladin. In mouse kidney sections and cultured mouse podocytes, they confirmed that palladin and synaptopodin colocalised in dense bodies along actin filaments and that palladin is present in focal adhesions. Knock-down of palladin in cultured podocytes was associated with a higher number of small focal adhesions, fewer actin fibres and a more migratory phenotype. Podocyte-specific knock-out in mice led to an enlarged sub-podocyte space, reduced expression of nephrin and increased proteinuria following injection of nephrotoxic serum compared with control mice. The expression of palladin was reduced in podocytes in renal biopsies from patients with diabetic nephropathy and FSGS. In a separate study, the same group also found that in the palladin knock-out mice the filtration slit

density was significantly lower than in control mice [393]. Taken together, these two studies highlight the importance of palladin for podocyte structure and function but neither these nor others in the published literature have examined the effects, or control, of palladin phosphorylation in these cells.

Other groups have sought to develop podocyte-based assays to quantify the effects of circulating factor activity. Kachurina *et al*. used ci-hPod treated for 20 hours with serum from 10 patients with FSGS: 4 had post-transplant recurrence and 4 did not; 2 patients had idiopathic, non-genetic FSGS and had not been transplanted [394]. By immunofluorescence and computerised image analysis, they calculated the number of focal adhesion complexes (FACs) per 1000 μm^2 cell area. In all patients with post-transplant recurrence and the 2 with idiopathic FSGS, the number of FACs decreased significantly compared with control whereas there was no difference when treated with serum from the patients with no recurrence. Pre-incubation of podocytes with anti-TNF receptor antibodies before addition of sera prevented the reduction in FACs in 2 of the 4 recurrent and both idiopathic FSGS cases. The authors suggested their assay may be able to identify patients who would respond to TNF-alpha blockade. They did not compare serial samples from the same patients at times of relapse and remission and the timing of serum samples with respect to transplantation was unclear. Further study using longitudinally-collected samples, particularly pre-transplant, would be important to evaluate the predictive value of this assay.

The current study examined the podocyte phosphoproteome after 30 minutes of treatment and that of Kachurina *et al*. assessed FACs at 20 hours. It is possible that phosphorylation of palladin and of VASP [188], with which it interacts [386, 395], are early steps in the pathway leading to reduction of FACs

in podocytes. In the future, development of an anti-phospho (Ser893)-palladin antibody would facilitate validation by Western blotting of the MS findings in this study. Immunofluorescence could also be used to visualise phospho-palladin and FACs after treatment of podocytes with relapse and remission plasma.

7.4.2 Limitations

Although this study has highlighted some important findings which will require further investigation, it has several limitations. The phosphoproteomic analysis included a relatively small number of patients and, therefore, results may not be reproduced in a larger cohort. The pathogenic processes and circulating factors may have been different between the patients although they were included as all had SRNS with post-transplant recurrence of disease. In this study, proteins were extracted from podocytes after 30 minutes of treatment with plasma. It therefore gives only a snapshot of the phosphoproteome and cannot provide information about differences in phosphorylation within the first minutes after treatment with relapse or remission plasma.

7.4.3 Conclusion

This study aimed to examine differences in phosphoproteins in podocytes after *in vitro* treatment with SRNS relapse and remission plasma. It highlighted that proteins involved in biochemical pathways related to actin cytoskeleton reorganisation and focal adhesion are differentially phosphorylated in podocytes exposed to the two treatments. It also pointed to palladin as a protein which was markedly more phosphorylated after treatment with relapse plasma. This study, however, was not able to validate the MS findings with an alternative technique

and this was limited by the lack of a specific phospho-palladin antibody. Given the increasing literature suggesting the importance of palladin in podocyte structure and function, future studies are required to examine the role of palladin phosphorylation in pathogenesis and as a biomarker in SRNS relapse.

Chapter 8 Discussion

SRNS is a rare disease affecting predominantly children, but also adults. Although absolute numbers are comparatively small, the impact on patients' lives, with the high likelihood of development of ESRF, requirement for dialysis or transplantation and associated mortality, is significant. It is clear that SRNS is not a single disease but a common endpoint for a heterogenous group of underlying genetic and non-genetic pathogenic processes. There is variation in the success of immunosuppressive treatments after the failure of steroids, and also variable outcome following transplantation, with up to 50% of patients suffering post-transplant disease recurrence [142].

The aim of this study was to investigate the interplay of phenotype, genotype and potential biomarkers with respect to treatment and long-term outcome. In particular, the intention was to develop further stratification of patients and identify biomarkers which could be used in future trials of treatments.

8.1 Findings of this study

The main findings of this study were:

- The pattern of steroid resistance strongly predicted non-genetic disease: no patients with secondary steroid resistance had a pathogenic variant found by whole exome sequencing.
- When considering first renal biopsies in SRNS, there was no significant difference in frequency of genetic disease in those with FSGS versus MCD.

- Patients with presumed steroid resistance were significantly more likely to progress to ESRF than patients with either primary or secondary steroid resistance (there was no significant difference between the latter two).
- Patients with genetic disease were significantly more likely to progress to ESRF, but none suffered post-transplant recurrence.
- Secondary steroid resistance was a strong predictor of post-transplant recurrence supporting previous findings [274].
- Pathogenic variants were detected in 21.2% of patients with SRNS by clinical genetic testing in a real-world international cohort and in 27.8% of children with SRNS by WES in a research cohort.
- Complete response to first IIS treatment was seen in only approximately 25% of patients with similar response rates for ciclosporin, tacrolimus and MMF.
- Only rituximab when used to treat patients with SSR had a higher complete response rate at 66.7%.
- Irrespective of the drug used, complete or partial response to the first-line IIS treatment was associated with a highly significantly lower risk of progression to ESRF compared with no response to first treatment.
- Plasma proteomics identified uteroglobin as a potential biomarker being raised at time of disease relapse versus remission, although further validation, and study with longitudinally-collected samples, is required to confirm whether it has any predictive value.
- Hemopexin, NGAL and AHSG, which have been suggested as potential biomarkers in other studies, also appeared to be differentially present in relapse versus remission plasma in this study.

- Phosphorylation of palladin in podocytes may be an important step in disease pathogenesis after exposure to relapse plasma based on phosphoproteomic studies and knowledge of palladin function from the published literature. This would be worthy of more detailed *in vitro*, and subsequently *in vivo*, study

Based on the findings from this study, a clinical diagnostic and management pathway can be proposed. Patients with presumed or primary steroid resistance could be offered clinical genetic testing using a gene panel approach from the point of diagnosis. From the results reported in Chapter 4 and other studies [44, 57, 396], a genetic cause is likely to be detected in 25-30%. Age of onset under 1 year, consanguinity and family history of NS do appear to be associated with greater risk of genetic disease, as would be expected. However, in this and other published studies, absence of these factors does not rule out a genetic basis for disease. This, therefore, supports universal testing in patients with presumed or primary SR. Others have also recommended clinical genetic testing using WES in all patients with SRNS with onset under 25 years [281, 396]. The caution against more prevalent genetic testing is the existence of variants of uncertain significance and incidental discovery of secondary variants in genes other than that thought to be primarily pathogenic [397]. These secondary variants may have modifier effects that are incompletely understood and are likely to complicate genetic counselling.

In this study, no patients with secondary SR had genetic disease. Therefore, in clinical practice, it may be reasonable not to undertake genetic testing in this subgroup, although a small number of patients with SSNS have

been identified with mutations in *EMP2* [108]. In the context of future research, particularly using WES or WGS, patients with secondary SR should still be included as novel gene associations may be discovered.

Following clinical genetic testing, those patients diagnosed with genetic disease could receive more specific counselling about prognosis, and it would also enable the patient's family to be informed of the likelihood of disease being inherited by future children. Although the chance of response to IIS treatment is lower and the risk of progression to ESRF is higher, as discussed in Chapter 3, some patients with genetic disease do respond to ciclosporin or tacrolimus [276, 277]. Furthermore, there have been published reports of patients with *WT1*, *NPHS1* or *NPHS2* variants responding to IIS therapy [82, 276]. Patients with SRNS and pathogenic variants in genes coding for proteins involved in the coenzyme Q_{10} pathway (such as *COQ2*, *COQ6*, *PDSS2* and *ADCK4*) have been shown to be successfully treated with coenzyme Q_{10} supplementation [119, 124, 301].

Further research and international sharing of data is required to determine if patients with specific variants in specific genes are more responsive to immunosuppression. The non-immunologic mechanisms of action of immunosuppressants, as discussed in Chapter 1 (Section 1.5.1), provide a rationale for their efficacy in genetic disease. Greater understanding of the pathogenic mechanisms of causative mutations will also open new doors for potential treatment such as gene therapy.

For those patients with genetic disease who do require transplantation, findings from the studies reported here support previous research that the likelihood of recurrence is close to zero [44]. The main exception, reported by

others, has been those patients with *NPHS1* variants leading to complete absence of nephrin in whom anti-nephrin antibodies are associated with post-transplant proteinuria [398]. The PodoNet cohort study also reported 4 patients with *NPHS2* variants who suffered post-transplant recurrence [44]. No anti-podocin antibodies have been detected and the mechanism of recurrence in this group of patients is unclear [399].

Patients with presumed or primary SR with single heterozygous variants in causative genes may still have genetic disease. As discussed in Chapter 4, second variants in intronic or promoter regions, or combinatorial effects with variants in other known or novel causative genes, may together be pathogenic. WGS and future functional studies will hopefully elucidate these hypotheses.

In those patients with presumed or primary SR and no genetic cause, IIS treatment should be the mainstay of treatment. This study has shown that tacrolimus and ciclosporin seem to have similar efficacy, but in both cases complete or partial response was under 50%. As a retrospective and non-controlled cohort study, the findings are limited due to potential confounding from concomitant treatments. Prospective randomised controlled trials of novel therapies in SRNS are required with better patient stratification [195].

Patients with secondary SR did show a higher response rate to rituximab (Chapter 3) and previous studies have shown evidence of efficacy in FR-SSNS and SDNS [206]. A systematic review of rituximab use in SRNS including 226 patients reported remission in 40.8% of those with initial SR and 52.8% with late SR [273]. Consideration should be given to targeting patients with secondary SR to earlier treatment with rituximab, although in many cases it may already have been given while the patient was steroid sensitive.

Early post-transplant disease recurrence, which is seen most frequently in patients with secondary SR, is an indicator of circulating factor disease. This study sought biomarkers in plasma by conducting proteomic analysis at the time of disease relapse and remission in patients who had had post-transplant recurrence. Uteroglobin was highlighted as a protein increased in relapse versus remission plasma reasonably consistently across patients (Chapter 5). However, as a steroid-inducible protein it may be confounded by treatment. The ideal biomarker would be a protein, or group of proteins, which can be measured longitudinally and has the ability to predict which patients will have post-transplant recurrence prior to the transplant taking place.

8.2 Limitations of this study

Specific limitations have been discussed in preceding chapters; however, there are several which have implications for the study as a whole. Given the opportunity to repeat the project from the start, there are different approaches which could be adopted to improve the quality and reliability of the conclusions.

The RaDaR cohort included patients retrospectively, in some cases years after the initial diagnosis of SRNS. It relied on clinicians and research nurses, often with many other commitments, inputting details from paper and electronic medical records. These factors probably contributed to missing demographic, clinical and follow-up data. A prospectively-collected cohort, including only incident cases, would, most likely, improve the completeness of the data. This would be further enhanced by direct linking of electronic patient records with the RaDaR database. During the course of this study, this link was established for some patients at certain hospital trusts which were using Renal Patient View. Had

this been in place for all patients, complete laboratory results would have been available, thus improving the quality of data for analysis of responses to treatments.

The investigation of potential biomarkers in SRNS in this study was dependent on biological samples from patients. Blood samples were sent to the University of Bristol at ambient temperature and in some cases arrived several days after leaving the patient. Haemolysis and protein degradation during this time was likely to have altered the plasma proteome. Furthermore, patients would have been receiving different treatments at the times of sampling. The effects of these confounding variables could have been reduced by obtaining samples at the time of diagnosis before any steroids, and by establishing local standard operating procedures for centrifuging the blood and freezing plasma within 30 minutes of sampling.

This study used plasma exchange fluid for proteomic analysis by mass spectrometry but was limited by the availability of paired samples at times of relapse and remission. The heterogeneity became more apparent during the course of the research, particularly relating to the timing of "relapse" and "remission" samples with respect to each other and renal transplantation. Sample collection and labelling were undertaken by local clinicians / research nurses and could have been improved with more stringent definitions and protocols.

The proteomics studies were limited by the heterogeneity of the samples, and the small number of patients included in the initial search for potential biomarkers. A future, expanded cohort with paired plasma samples and complete clinical and laboratory data would facilitate selection of a larger group of more homogeneous patients.

8.3 Future studies

As a rare disease, continued expansion of the national cohort with high-quality baseline and follow-up data is essential as a basis for future research. Obtaining DNA samples for genetic analysis soon after diagnosis and biological samples (urine, blood plasma and, when performed, renal biopsy) longitudinally ideally prior to any IIS treatments, at times of relapse and remission, and before and after transplant will enhance the ability to identify and validate predictive biomarkers. The RaDaR and NephroS studies have continued to expand and Bristol Renal has taken a leading role in the development of NURTuRE (National Unified Renal Translational Research Enterprise), a biobank for CKD and INS covering England, Scotland and Wales [400]. The aim will be to include 800 adults and children with INS.

Genetic analyses discussed in this study have focussed on WES in a research cohort and gene panel testing in the clinical context. As noted in Chapters 2 and 4, there were some patients with early-onset disease or CNS with no or single heterozygous recessive variants in known causative genes. Future investigation of these patients, in particular, with WGS will help to identify novel genes or variants in intronic regulatory elements which may be pathogenic. An understanding of how these cause disease may offer new opportunities to develop targeted treatments. For example, identification of variants in 6 genes coding for proteins involved in RhoA regulation in families with partially treatment-sensitive NS suggests the Rho GTPase pathway may be a potential therapeutic target [401].

Given the relatively low complete response rate to older ISS agents, novel therapies to treat SRNS are required. In order to interpret and gain the most

benefit from future clinical trials, it will be essential that patients are stratified, either using inclusion criteria or by prospectively-defined subgroups, particularly on the basis of genetic/non-genetic diagnosis and pattern of steroid resistance. Two clinical trials are currently, or will shortly be, recruiting focussing on prevention of FSGS recurrence post-transplant. Both exclude patients with known genetic disease. The PRI-VENT FSGS study will randomise patients to receive pre-emptive rituximab versus placebo immediately prior to transplant [402]. The other study compares bleselumab (a fully human anti-CD40 monoclonal antibody) with MMF both alongside standard-of-care renal transplant immunosuppression [403].

Further identification and validation of plasma biomarkers to predict post-transplant disease relapse will be facilitated by NURTuRE. Samples soon after diagnosis, ideally prior to starting IIS treatment, and before transplant will enable analysis of the predictive capability of potential biomarkers. Likewise, additional longitudinal samples, taken at times of relapse and remission in patients who subsequently suffer post-transplant recurrence, may help to develop podocyte-derived biomarkers of circulating factor disease.

8.4 Conclusions

This study has highlighted the heterogeneity between patients with the diagnosis of SRNS which makes it a challenge both to study and to manage in clinical practice. The findings indicate that early genetic testing has the potential to provide a genetic diagnosis in around 25% of patients with subsequent implications for treatment decisions, transplantation and prognosis. The pattern of steroid resistance is an important factor: in particular, secondary SR was not

associated with genetic disease but did predict higher post-transplant recurrence in the small number of patients who progressed to ESRF. This group also showed better response to rituximab than other patients. Among patients who received IIS treatment, any response (complete or partial) was associated with a significantly lower risk of ESRF compared with those who showed no response. This study highlighted uteroglobin as a protein increased in plasma at time of relapse versus remission. Its potential as a biomarker will need to be evaluated in a larger group of patients with longitudinal sampling. Phosphorylation of palladin within podocytes, at an early stage after exposure to relapse plasma, may also have a role as a biomarker of circulating factors, but further *in vitro* work is required to support initial findings.

The next steps will be to develop clinical trials using genetic and phenotypic characteristics to stratify patients and enable appropriate subgroup analysis. In addition, longitudinal collection of biological samples should be integral to such trials to facilitate further biomarker identification and validation. Improving outcomes with targeted treatment, ensuring the right patient receives the right drug at the right time, will be the ultimate goal.

9 References

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10 Appendices

10.1 Demographic and clinical features of 187 patients who had whole exome sequencing (Chapter 2)

Table 10.1: Demographic and clinical features of 187 patients who had whole exome sequencing

Patient	Gene	Gender	Ethnicity	Familial / Sporadic	Age at onset (years)	Consan-guinity	Resistance to steroids	1st biopsy	latest biopsy	CKD stage	Time to ESRF	Transplanted?	Disease recurrence?	Extra-renal phenotype	Length of follow up (years)
562	No	F	P	F	0.0	Yes	presumed	ND	ND	5HD	0.7	No	N/A	Short bowel syndrome, cardiovascular disorder, imperforate anus, short gut syndrome, developmental delay	3.0
760	No	M	W	F	0.2	No	presumed	ND	ND	1	N/A	No	N/A	No	3.3
651	No	F	Mixed	S	0.6	No	presumed	DMS	ND	2	N/A	No	N/A	No	14.4
377	No	F	P	S	1.0	Yes	primary	Other	ND	1	N/A	No	N/A	No	4.7
286	No	F	W	S	1.1	No	primary	FSGS	ND	1	N/A	No	N/A	No	7.6
509	No	M	W	S	1.2	No	primary	Other	Other	1	N/A	No	N/A	Atopic Eczema	4.6
608	No	F	W	S	1.2	No	primary	FSGS	FSGS	1	N/A	No	N/A	No	11.8
435	No	F	W	S	1.3	No	primary	FSGS	ND	1	N/A	No	N/A	Probable Crohn's disease	12.2
356	No	M	Mixed B	F	1.3	No	secondary	MCD	ND	5Tx	8.2	Yes	Yes	No	15.5
400	No	M	P	S	1.4	Yes	primary	DMS	DMS	5Tx	0.8	Yes	No	Developmental delay with microcephaly, Diabetes	9.0
469	No	M	W	S	1.5	No	secondary	FSGS	MCD	1	N/A	No	N/A	No	15.2
314	No	F	W	S	1.6	No	primary	MCD	FSGS	1	N/A	No	N/A	No	6.6
412	No	M	W	S	1.7	No	primary	MHc	MHc	1	N/A	No	N/A	No	11.9
443	No	M	W	S	1.7	Unknown	primary	MCD	FSGS	1	N/A	No	N/A	No	16.3
502	No	F	I	S	1.7	No	primary	MCD	MCD	1	N/A	No	N/A	No	5.9
787	No	M	W	S	1.7	No	sensitive (frequently relapsing)	FSGS	ND	1	N/A	No	N/A	No	3.4
361	No	M	W	S	1.8	No	primary	FSGS	MHc	1	N/A	No	N/A	Asthma	8.2

730	No	M	B	S	1.8	No	primary	MCD	MCD	5PD	1.1	No	N/A	No	4.4
464	No	M	Ban	S	1.9	No	secondary	FSGS	ND	5Tx	9.4	Yes	No	No	16.5
713	No	M	W	S	2.0	No	secondary	FSGS	FSGS	1	N/A	No	N/A	No	9.0
732	No	M	I	S	2.0	No	primary	FSGS	ND	1	N/A	No	N/A	Purpura, haematuria	1.3
401	No	M	W	S	2.1	No	primary	Other	ND	1	N/A	No	N/A	No	8.2
490	No	F	W	S	2.1	No	primary	FSGS	FSGS	5Tx	1.4	Yes	No	No	5.2
425	No	M	W	S	2.1	No	primary	ND	Other	2	N/A	No	N/A	No	5.4
680	No	F	W	S	2.2	No	primary	MCD	ND	1	N/A	No	N/A	No	3.0
329	No	M	W	S	2.2	No	primary	FSGS	FSGS	1	N/A	No	N/A	No	8.0
444	No	M	W	S	2.2	No	primary	FSGS	ND	5Tx	0.1	Yes	No	No	12.7
394	No	F	W	S	2.2	No	primary	MCD	FSGS	5Tx	2.0	Yes	Yes	No	5.0
452	No	F	W	S	2.2	No	primary	FSGS	ND	5Tx	0.8	Yes	No	Motor delay	12.0
312	No	F	W	S	2.2	No	primary	FSGS	ND	5Tx	2.9	Yes	No	No	11.4
508	No	M	W	S	2.3	No	primary	MCD	MCD	1	N/A	No	N/A	No	6.8
287	No	M	Mixed (W and B)	S	2.3	No	primary	MCD	FSGS	5Tx	2.9	Yes	Yes	No	9.8
712	No	M	Mixed	S	2.4	No	secondary	FSGS	ND	1	N/A	No	N/A	No	14.1
505	No	F	W	S	2.4	No	secondary	TBMN	ND	1	N/A	No	N/A	No	5.2
703	No	M	W	S	2.4	No	primary	Other	MHc	1	N/A	No	N/A	No	4.7
533	No	M	W	S	2.4	No	primary	MCD	ND	1	N/A	No	N/A	No	3.1
351	No	M	P	S	2.4	No	primary	FSGS	CNI toxicity	1	N/A	No	N/A	No	10.9
567	No	M	As	S	2.5	No	secondary	MCD	MCD	1	N/A	No	N/A	No	9.4
324	No	F	W	S	2.6	No	secondary	FSGS	ND	1	N/A	No	N/A	Seizure activity	7.5
480	No	F	B	S	2.6	No	primary	MHc	FSGS	1	N/A	No	N/A	No	6.4
439	No	F	Other	S	2.6	No	primary	FSGS	ND	1	N/A	No	N/A	No	6.5
682	No	M	As	S	2.7	No	secondary	FSGS	ND	1	N/A	No	N/A	Hypercholesteremia, auto-immune disease	4.2
448	No	M	P	S	2.7	No	primary	FSGS	ND	5Tx	0.5	Yes	Yes	No	16.0
526	No	M	W	S	2.8	No	primary	MCD	ND	1	N/A	No	N/A	Ex-34 weeks premature, global developmental delay	3.0
357	No	M	I	F	3.0	No	primary	FSGS	ND	5Tx	2.0	Yes	Yes	No	11.8
532	No	M	W	S	3.0	No	primary	FSGS	ND	5Tx	5.4	Yes	No	No	16.6
284	No	M	W	S	3.0	No	primary	MHc	ND	5PD	9.9	No	N/A	No	10.7
513	No	M	P	S	3.0	No	primary	FSGS	ND	3	N/A	No	N/A	No	5.4

460	No	F	W	S	3.2	No	primary	FSGS	FSGS	1	N/A	No	N/A	No		4.5
735	No	M	W	S	3.2	No	primary	Other	ND	1	N/A	No	N/A	No		3.4
393	No	F	B	S	3.3	No	primary	FSGS	MCD	1	N/A	No	N/A	No		10.0
437	No	F	W	S	3.4	No	primary	FSGS	ND	1	N/A	No	N/A	No		6.7
420	No	M	Other	S	3.4	No	primary	FSGS	ND	5Tx	0.0	Yes	No	No		14.6
450	No	F	Mixed	S	3.5	No	primary	FSGS	FSGS	1	N/A	No	N/A	No		6.4
403	No	F	B	S	3.5	No	secondary	FSGS	ND	1	N/A	No	N/A	No		5.8
415	No	F	W	S	3.6	No	primary	FSGS	FSGS	1	N/A	No	N/A	No		8.0
436	No	F	B	S	3.6	No	primary	FSGS	ND	1	N/A	No	N/A	No		10.7
451	No	F	W	S	3.7	No	secondary	FSGS	ND	5Tx	2.1	Yes	Yes	No		13.1
445	No	M	B	S	3.8	No	primary	FSGS	ND	5Tx	4.5	Yes	No	No		11.9
309	No	F	I	S	3.9	No	primary	MCD	ND	1	N/A	No	N/A	No		2.2
427	No	M	W	S	3.9	No	secondary	FSGS	ND	5PD	3.6	No	N/A	No		3.8
413	No	M	P	S	4.0	No	secondary	MCD	FSGS	1	N/A	No	N/A	No		6.2
449	No	F	W	S	4.0	No	primary	FSGS	ND	5Tx	8.0	Yes	No	No		14.0
546	No	M	W	S	4.1	No	primary	MCD	Other	1	N/A	No	N/A	No		12.2
344	No	M	Ban	S	4.1	Unknown	primary	MHc	MHc	1	N/A	No	N/A	No		15.2
668	No	F	W	S	4.1	No	primary	FSGS	ND	1	N/A	No	N/A	No		8.0
552	No	M	W	F	4.2	Yes	secondary	MCD	ND	1	N/A	No	N/A	No		10.2
402	No	M	W	S	4.3	No	secondary	MCD	ND	1	N/A	No	N/A	No		3.7
489	No	F	As	F	4.4	Unknown	primary	FSGS	ND	1	N/A	No	N/A	No		4.1
326	No	M	W	S	4.5	No	secondary	FSGS	ND	1	N/A	No	N/A	No		9.9
346	No	F	W	S	4.6	No	primary	FSGS	ND	5Tx	4.6	Yes	Yes	No		15.5
363	No	F	B	S	4.7	No	secondary	MCD	FSGS	4	N/A	No	N/A	No		15.9
528	No	F	W	S	4.7	No	primary	FSGS	ND	1	N/A	No	N/A	No		3.3
473	No	M	W	S	4.8	No	primary	MCD	ND	5ND	5.1	No	N/A	No		5.5
685	No	F	P	S	4.8	No	primary	FSGS	ND	1	N/A	No	N/A	No		3.1
347	No	F	W	S	4.9	No	primary	FSGS	ND	1	N/A	No	N/A	No		2.1
534	No	F	W	S	5.0	No	primary	MHc	FSGS	1	N/A	No	N/A	No		10.4
366	No	M	W	F	5.1	No	primary	FSGS	ND	5Tx	6.7	Yes	Yes	No		15.2
488	No	M	W	S	5.2	No	primary	FSGS	FSGS	1	N/A	No	N/A	Hypertension		8.2
342	No	F	As	S	5.4	No	primary	FSGS	ND	1	N/A	No	N/A	No		5.2
620	No	F	Mixed	S	5.6	No	not tried	FSGS	ND	1	N/A	No	N/A	No		11.8
434	No	M	W	S	5.6	No	primary	MHc	MCD	1	N/A	No	N/A	No		6.3
355	No	F	W	S	5.6	No	primary	FSGS	ND	1	N/A	No	N/A	No		4.0
349	No	M	W	S	5.9	No	primary	FSGS	ND	2	N/A	No	N/A	No		5.7
453	No	M	W	S	5.9	No	secondary	FSGS	FSGS	5Tx	9.7	Yes	No	No		16.2
411	No	M	W	S	5.9	No	primary	FSGS	ND	5Tx	0.6	Yes	No	Short stature		15.0
486	No	F	W	S	6.2	No	primary	MCD	CNI toxicity	1	N/A	No	N/A	Hypo-gammaglobulinaemia, eczema	asthma,	5.2
615	No	F	W	F	6.3	No	secondary	MCD	ND	5HD	2.1	No	N/A	Cataract, diplopia		4.7
360	No	F	W	S	6.3	No	primary	MCD	ND	1	N/A	No	N/A	No		9.1
365	No	F	W	S	6.7	No	primary	FSGS	ND	1	N/A	No	N/A	No		3.4
728	No	M	W	S	6.7	No	primary	FSGS	ND	1	N/A	No	N/A	No		4.3

561	No	M	W	S	7.0	No	primary	MCD	ND	5Tx	2.2	Yes	Yes	Bicuspid aortic valve with mild incompetence	5.7
457	No	F	W	S	7.2	No	secondary	MCD	ND	1	N/A	No	N/A	No	7.2
392	No	F	Mixed B	S	7.3	No	sensitive	MCD	FSGS	1	N/A	No	N/A	No	5.7
641	No	F	P	S	7.8	Unknown	primary	MCD	FSGS	5Tx	1.8	Yes	Yes	Short stature	4.2
419	No	M	W	S	8.0	No	primary	MCD	FSGS	5Tx	5.2	Yes	Yes	No	8.7
410	No	F	W	S	8.2	No	primary	FSGS	FSGS	1	N/A	No	N/A	No	7.7
749	No	M	W	Unknown	8.2	Unknown	primary	FSGS	ND	1	N/A	No	N/A	No	1.4
417	No	F	Mixed	S	8.7	No	secondary	FSGS	ND	1	N/A	No	N/A	No	7.5
558	No	M	W	S	8.8	No	primary	MCD	MCD	1	N/A	No	N/A	No	4.9
466	No	M	W	S	8.9	No	secondary	MCD	ND	5Tx	3.0	Yes	Yes	No	8.9
432	No	M	W	S	8.9	No	secondary	FSGS	ND	1	N/A	No	N/A	No	3.5
294	No	F	W	S	8.9	No	primary	FSGS	ND	5Tx	1.9	Yes	Yes	No	16.2
276	No	M	As	S	9.2	No	primary	FSGS	FSGS	5Tx	3.6	Yes	Yes	Dyslexia	7.8
506	No	F	W	S	9.3	No	primary	FSGS	FSGS	1	N/A	No	N/A	No	4.4
629	No	F	W	S	9.3	No	primary	MCD	FSGS	1	N/A	No	N/A	No	3.2
291	No	F	W	S	9.5	No	primary	FSGS	ESRF	5Tx	5.5	Yes	Yes	No	12.9
475	No	F	W	S	9.6	No	secondary	MCD	MCD	1	N/A	No	N/A	No	4.1
754	No	M	W	S	9.8	No	primary	FSGS	ND	1	N/A	No	N/A	No	2.6
525	No	F	W	S	9.9	No	primary	ND	ND	2	N/A	No	N/A	Asthma	4.3
556	No	M	W	S	10.0	No	primary	FSGS	ND	1	N/A	No	N/A	No	5.1
654	No	F	W	S	10.5	No	primary	FSGS	ND	2	N/A	No	N/A	No	4.3
390	No	F	Other	S	10.5	No	secondary	MCD	ND	1	N/A	No	N/A	No	2.4
316	No	M	W	S	11.3	No	primary	FSGS	ND	5Tx	2.2	Yes	Yes	No	9.5
385	No	M	W	S	11.3	No	primary	FSGS	ND	1	N/A	No	N/A	No	5.0
441	No	M	W	S	11.4	No	primary	FSGS	ND	1	N/A	No	N/A	Hodgkin's lymphoma	6.5
395	No	M	I	S	11.7	No	secondary	MHc	FSGS	5Tx	3.8	Yes	Yes	No	6.9
440	No	M	W	S	12.0	No	primary	Other	ND	1	N/A	No	N/A	No	8.2
389	No	F	I	S	12.1	No	secondary	FSGS	ND	5HD	4.7	No	N/A	Developmental delay, severe learning difficulties, coeliac disease	5.0
560	No	F	I	S	12.1	No	primary	FSGS	ND	5Tx	1.9	Yes	Yes	No	5.2
767	No	M	W	S	12.2	No	primary	FSGS	ND	1	N/A	No	N/A	No	2.6
298	No	M	W	S	12.4	No	primary	FSGS	ND	5Tx	4.3	Yes	No	No	5.9
345	No	F	W	S	12.4	No	primary	FSGS	ND	1	N/A	No	N/A	No	5.8
689	No	F	W	S	12.7	No	primary	FSGS	ND	1	N/A	No	N/A	No	4.0
372	No	M	W	S	12.8	No	primary	FSGS	ND	1	N/A	No	N/A	Psoriasis	9.0
494	No	F	W	S	12.9	No	primary	FSGS	ND	1	N/A	No	N/A	No	8.6
650	No	F	Mixed (W and As)	S	13.2	No	primary	MCD	ND	1	N/A	No	N/A	No	2.4
649	No	M	W	S	13.4	No	sensitive	FSGS	ND	1	N/A	No	N/A	No	4.1
330	No	F	W	S	13.8	No	primary	MHc	ND	4	N/A	No	N/A	No	8.3
671	No	M	W	S	13.9	No	primary	MCD	FSGS	4	N/A	No	N/A	No	4.6
507	No	M	As	S	14.6	Yes	presumed	FSGS	FSGS	5HD	2.0	No	N/A	Asthma	4.3
605	No	F	W	S	15.1	No	primary	FSGS	ND	5ND	5.1	No	N/A	No	6.0

295	No	F	W	S	15.2	No	primary	FSGS	FSGS	5PD	2.8	No	N/A	No	3.9
258	No	F	W	S	15.4	No	primary	FSGS	ND	1	N/A	No	N/A	Asthma	2.4
630	No	F	B	S	16.1	No	primary	FSGS	ND	5Tx	1.2	Yes	No	No	4.4
821	*NPHS1*	M	W	S	0.0	No	presumed	ND	ND	5Tx	2.3	Yes	No	Hypothyroidism	5.7
478	*NPHS1*	M	W	S	0.0	No	presumed	MCD	ND	5Tx	0.5	Yes	No	Epilepsy, ADHD, short stature,	18.9
315	*NPHS1*	M	W	S	0.0	No	presumed	Finnish	Finnish	5Tx	1.3	Yes	No	Communicating hydrocephalus	8.5
302	*NPHS1*	M	I	S	0.1	Yes	presumed	DMS	DMS	5Tx	3.3	Yes	No	No	7.9
430	*WT1*	F	W	S	0.1	No	presumed	MCD	ND	5Tx	0.0	Yes	No	ophthalmic problems, no gonadoblastoma, no Wilm's tumour, normal 46XX	21.7
386	*NPHS1*	M	W	S	0.1	No	presumed	Finnish	ND	5Tx	4.7	Yes	No	No	10.9
375	*NPHS1*	F	P	S	0.1	Yes	presumed	Other	ND	5Tx	1.9	Yes	No	Renal extramedullary haematopoiesis, mild pulmonary stenosis and VSD	11.1
503	*LAMB2*	M	W	S	0.1	No	presumed	ND	ND	5PD	0.0	No	N/A	Pierson syndrome	5.6
431	*NPHS1*	M	W	S	0.1	No	presumed	Other	ND	5Tx	1.8	Yes	No	No	7.0
353	*NPHS1*	M	mixed (W and B)	S	0.1	No	sensitive (frequently relapsing)	Other	ND	1	N/A	No	N/A	No	17.7
305	*NPHS1*	M	W	S	0.1	No	presumed	MCD	ND	5Tx	1.6	Yes	No	No	15.2
621	*NPHS1*	M	I	S	0.1	No	presumed	Finnish	Finnish	5Tx	4.4	Yes	No	developmental delay	8.1
497	*NPHS1*	M	W	S	0.2	No	primary	Finnish	ND	5Tx	2.3	Yes	No	No	13.6
731	*MAGI2*	M	W	S	0.3	No	primary	MCD	ND	1	N/A	No	N/A	Pyloric stenosis, Polydactyly, Thrombocytosis	11.7
704	*MAGI2*	F	W	F	0.4	No	presumed	Finnish	Finnish	5Tx	0.5	Yes	No	No	11.5
652	*COL4A5*	M	P	F	0.5	Yes	primary	Other	ND	5PD	2.2	No	N/A	No	3.3
687	*CRB2*	F	W	S	0.8	No	presumed	MCD	ND	1	N/A	No	N/A	No	4.0
442	*MYO1E*	F	Other	S	1.0	Yes	primary	ND	ND	5PD	4.1	No	N/A	No	6.0
297	*NPHS2*	M	W	S	1.0	No	primary	FSGS	ND	5Tx	7.6	Yes	No	No	14.9
285	*WT1*	F	W	S	1.0	No	presumed	ESRF	ND	5Tx	0.0	Yes	No	Klinefelter's syndrome - XXY, Gonadoblastoma	11.0
311	*NPHS2*	F	W	F	1.1	No	presumed	MHc	ND	5ND	11.6	No	N/A	No	11.7
352	*NPHS1*	F	W	S	1.4	No	presumed	ESRF	ND	5Tx	2.4	Yes	No	No	16.0
319	*NPHS2*	M	W	S	1.8	No	primary	FSGS	FSGS	5Tx	7.5	Yes	No	No	10.4
496	*WT1*	F	W	S	1.9	No	primary	FSGS	ND	5Tx	9.0	Yes	No	Normal 46XX	10.7
495	*NPHS1*	F	Other	S	2.0	No	primary	MCD	MCD	2	N/A	No	N/A	No	17.8
467	*NPHS2*	F	W	S	2.0	No	primary	MCD	FSGS	5Tx	3.3	Yes	No	No	6.8
323	*NUP107*	M	P	S	2.1	Yes	primary	FSGS	ND	5Tx	0.5	Yes	No	Developmental delay	5.8
678	*NPHS2*	M	W	S	2.8	No	primary	ND	MCD	5Tx	2.4	Yes	No	No	15.9
683	*DGKE*	F	W	S	3.1	No	primary	FSGS	ND	1	N/A	No	N/A	Dyslexia	14.9
296	*TRPC6*	M	W	S	3.2	No	primary	MCD	ND	1	N/A	No	N/A	No	5.0
383	*NPHS2*	F	W	S	3.7	No	primary	FSGS	ND	5Tx	4.4	Yes	No	No	14.0
811	*WT1*	F	W	S	3.8	No	primary	Alport	ND	1	N/A	No	N/A	Chronic cough and diarrhoea, Frasier syndrome, XY**	1.1
414	*NPHS1*	M	W	F	5.7	Unknown	primary	MHc	MHc	1	N/A	No	N/A	Asthma, eczema	11.3

446	*NPHS2*	F	P	F	6.1	No	primary	FSGS	ND	5Tx	2.2	Yes	No	No	10.9
388	*NUP93*	F	W	S	6.1	No	primary	FSGS	ND	5Tx	0.2	Yes	No	No	8.4
609	*NUP107*	M	P	F	6.1	Yes	presumed	ND	ND	1	N/A	No	N/A	Learning difficulties, eczema, microcephaly	6.7
492	*NPHS2*	F	W	F	7.0	No	primary	ND	ND	1	N/A	No	N/A	No	6.7
317	*TRPC6*	F	W	S	7.8	No	presumed	FSGS	ND	5Tx	0.0	Yes	No	No	7.4
770	*COL4A3*	F	W	S	7.9	No	primary	FSGS	ND	1	N/A	No	N/A	No	2.5
729	*NPHS2*	M	W	S	7.9	No	primary	Other	ND	2	N/A	No	N/A	Asthma	2.8
370	*APOL1*	M	B	S	9.9	No	primary	FSGS	ND	5HD	7.4	No	N/A	No	8.1
493	*NPHS2*	F	W	F	10.1	No	primary	FSGS	ND	5Tx	2.0	Yes	No	No	9.4
387	*ADCK4*	M	W	S	10.9	No	primary	FSGS	ND	5Tx	1.8	Yes	No	No	9.2
397	*ACTN4*	M	W	S	12.3	No	primary	Other	ND	5Tx	1.4	Yes	No	Aarskog Syndrome and Horseshoe Kidney	7.4
566	*NUP107*	F	P	F	12.6	Yes	presumed	ESRF	ND	5HD	0.0	No	No	Microcephaly	4.3
399	*OCRL*	M	W	F	12.6	No	primary	FSGS	ND	3	N/A	No	N/A	Short stature	10.0
516	*LMX1B*	F	W	F	13.1	No	presumed	ND	FSGS	1	N/A	No	N/A	Craniosynostosis, brachycephaly, fixed flexion deformities, short tibia, abnormal calf muscles, facial dysmorphism, receptive and expressive speech disorder	5.0
747	*NPHS2*	M	W	S	13.8	No	primary	FSGS	ND	5PD	1.3	No	N/A	No	3.9
672	*NPHS2*	F	W	F	14.1	No	primary	MCD	ND	1	N/A	No	N/A	Learning disabilities, pulmonary embolus	3.7
613	*NUP107*	F	P	F	14.2	Yes	presumed	FSGS	ND	1	N/A	No	N/A	Learning difficulties, eczema, microcephaly	2.2
900	*LMX1B*	F	W	F	14.9	No	primary	FSGS	ND	1	N/A	No	N/A	Delayed puberty	1.1
407	*PODXL*	M	W	S	15.2	No	primary	FSGS	ND	1	N/A	No	N/A	No	2.3

The table is sorted first by non-genetic / genetic status and then by age of onset.

Legend: ADHD, attention deficit hyperactivity disorder; As, Asian; B, Black African/Caribbean; Ban, Bangladeshi; CKD, chronic kidney disease; CNI, calcineurin inhibitor; CNS, Congenital nephrotic syndrome; DMH, Diffuse mesangial hypercellularity; DMS, Diffuse mesangial sclerosis; DPGN, Diffuse mesangial proliferative glomerulonephritis; ESRF, End stage renal failure; F, female (gender) / familial; FGGS, Focal global glomerulosclerosis; FSGS, Focal segmental glomerulosclerosis; HD, Haemodialysis; I, Indian; M, male; MCD, Minimal Change Disease; MHc, Mesangial hypercellularity; N/A, not applicable; ND, not done/no data available; P, Pakistani; PD, Peritoneal dialysis; S, sporadic; TBMN, thin basement membrane nephropathy; Tx, transplanted; VSD, ventricular septal defect; W, White; ** - karyotyping not done; NGS data analysis indicates XY

10.2 Demographic and clinical features of 63 patients who had clinical genetic testing (Chapter 3)

Table 10.2: Demographic and clinical features of 63 patients who had clinical genetic testing

Patient #	Gene	Gender	Ethnicity	Familial / Sporadic	Age at onset(years)	Consanguinity	Resistance to steroids	1st biopsy	CKD stage	Time to ESRF	Transplanted?	Disease recurrence?	Extra-renal phenotype	Length of follow up (years)
5288	No	F	Other	ND	0.0	ND	presumed	ND	1	N/A	No	N/A	No	1.6
2935	No	F	W	F	0.1	No	presumed	ND	1	N/A	No	N/A	Cardiac abnormality - PFO	3.8
424	No	M	W	S	0.1	No	presumed	Other	3	N/A	No	N/A	No	4.4
2015	No	M	As	F	0.2	Yes	proteinuria + FH	ND	1	N/A	No	N/A	No	4.1
2013	No	F	As	F	0.2	Yes	presumed	ND	1	N/A	No	N/A	No	0.8
9152	No	F	ND	ND	0.2	ND	presumed	ND	1	N/A	No	N/A	No	0.2
2249	No	F	As	S	0.3	No	presumed	ND	1	N/A	No	N/A	No	0.1
2014	No	F	As	F	0.6	Yes	proteinuria + FH	MHc	1	N/A	No	N/A	No	6.3
5000	No	F	ND	F	1.2	Yes	primary	FSGS	5HD	0.5	No	N/A	No	1.7
2755	No	M	W	S	1.9	No	secondary	Other	4	N/A	No	N/A	No	2.9
5939	No	M	W	S	2.2	No	secondary	ND	ND	N/A	ND	ND	No	0.0
7440	No	M	ND	ND	2.2	ND	primary	Other	ND	N/A	ND	ND	No	0.0
4327	No	F	W	F	2.4	No	secondary	ND	3	N/A	No	N/A	No	1.6
1412	No	M	W	S	2.9	No	primary	FSGS	1	N/A	No	N/A	No	2.5
2017	No	M	W	ND	3.0	ND	primary	FGGS	5ND	2.3	No	N/A	Developmental delay	2.4
622	No	M	As	S	3.1	No	primary	FSGS	1	N/A	No	N/A	No	4.6
11247	No	F	ND	F	3.2	No	primary	MCD	1	N/A	No	N/A	No	0.4
7277	No	M	W	ND	3.7	ND	presumed	ND	ND	N/A	ND	ND	No	21.0
5386	No	M	ND	ND	3.7	ND	primary	FSGS	ND	N/A	ND	ND	No	0.5
1191	No	F	W	S	3.8	No	primary	MCD	5HD	1.4	No	N/A	No	2.9
915	No	M	W	S	4.1	No	primary	FSGS	1	N/A	No	N/A	No	2.6
2250	No	F	W	S	4.1	No	primary	FSGS	1	N/A	No	N/A	No	1.7
2384	No	M	W	S	4.1	No	primary	FSGS	5Tx	1.3	Yes	Yes	No	2.9
2959	No	M	W	S	4.7	Yes	primary	FSGS	1	N/A	No	N/A	No	2.1
5912	No	F	W	S	4.7	No	primary	FSGS	1	N/A	No	N/A	No	1.5
5792	No	M	W	S	5.0	No	primary	MCD	1	N/A	No	N/A	No	0.1
2800	No	M	W	S	5.1	No	primary	FSGS	5HD	1.1	No	N/A	Cerebellar hypoplasia,	2.2

													developmental delay	
2908	No	F	As	S	6.9	No	primary	FSGS	5HD	9.1	No	N/A	No	9.4
866	No	M	B	S	7.2	No	primary	MCD	1	N/A	No	N/A	No	0.8
1108	No	F	W	S	8.7	No	primary	FSGS	1	N/A	No	N/A	No	1.2
242	No	M	W	S	10.2	No	primary	FSGS	5Tx	2.5	Yes	Yes	Aarskog-Scott Syndrome	12.5
293	No	F	W	S	10.3	No	primary	FSGS	1	N/A	No	N/A	No	6.6
1715	No	M	W	S	11.0	No	primary	Collapsing glomerulopathy	5Tx	0.3	Yes	Yes	ADHD	2.1
2772	No	F	W	S	11.6	No	primary	FSGS	1	N/A	No	N/A	No	0.1
273	No	M	W	S	12.0	No	primary	FSGS	5Tx	0.8	Yes	No	Unbalanced chromosome translocation	9.3
1291	No	F	W	S	12.4	No	primary	FSGS	5Tx	1.3	Yes	Yes	No	3.0
926	No	M	As	S	13.4	No	primary	FSGS	3	N/A	No	N/A	No	3.3
1202	No	M	W	F	13.6	No	secondary	FSGS	5Tx	0.9	Yes	Yes	Adrenal insufficiency	5.0
3242	No	F	W	S	14.0	No	primary	FSGS	1	N/A	No	N/A	No	0.9
8071	No	M	W	S	14.0	No	primary	FSGS	1	N/A	No	N/A	No	0.6
2107	No	F	W	S	14.3	No	primary	FSGS	1	N/A	No	N/A	No	1.9
2388	No	M	W	S	15.7	No	primary	FSGS	5ND	2.1	No	N/A	No	2.7
3604	*NPHS1*	M	ND	ND	0.0	ND	presumed	ND	1	N/A	No	N/A	No	0.7
3988	*NPHS1*	M	W	F	0.0	No	presumed	ND	1	N/A	No	N/A	No	2.0
3989	*NPHS1*	F	W	ND	0.0	ND	presumed	ND	5Tx	2.8	Yes	No	No	5.2
406	*NPHS1*	F	B	F	0.0	Yes	presumed	MHc	1	N/A	No	N/A	No	10.8
12156	*NPHS1*	F	W	S	0.0	No	presumed	ND	ND	N/A	ND	ND	No	0.0
476	*NPHS1*	M	W	ND	0.0	ND	presumed	ESRF	5Tx	2.1	Yes	No	No	5.5
12011	*NPHS1*	M	W	ND	0.0	ND	presumed	ESRF	5Tx	2.3	Yes	No	No	9.0
281	*NPHS1*	F	W	S	0.0	No	presumed	ND	5Tx	7.1	Yes	No	Possible autoimmune disease	10.0
3434	*NPHS1*	F	ND	S	0.1	No	presumed	ND	1	N/A	No	N/A	No	0.6
8903	*NPHS1*	F	As	S	0.1	Yes	presumed	ND	5Tx	3.9	Yes	No	Neutropenia	7.4
3128	*NPHS1*	M	ND	ND	0.1	ND	presumed	ND	5Tx	12.9	Yes	No	No	17.4
1810	*NPHS1*	M	As	S	0.1	Yes	presumed	ND	ND	N/A	ND	ND	No	1.7
405	*NPHS1*	F	B	F	0.2	Yes	presumed	MHc	5Tx	13.1	Yes	No	No	14.4
5750	*PLCE1*	F	ND	S	1.1	Yes	presumed	FGGS	5PD	0.1	No	N/A	No	0.9
374	*NPHS1*	F	W	S	1.3	No	presumed	MHc	5Tx	2.1	Yes	No	No	4.4
7656	*WT1*	M	W	ND	3.2	ND	primary	DMS	2	N/A	No	N/A	Denys-Drash syndrome	1.6
343	*NPHS2*	F	ND	F	6.8	Yes	primary	MCD	1	N/A	No	N/A	No	7.0
514	*SMARCAL1*	M	W	S	7.1	No	primary	FSGS	1	N/A	No	N/A	No	0.4
2154	*NPHS2*	F	As	F	9.2	No	presumed	ND	1	N/A	No	N/A	MPS type VI	5.5
9983	*NPHS2*	F	W	ND	11.7	ND	primary	FSGS	1	N/A	No	N/A	No	2.4
368	*NPHS1*	F	ND	ND	12.8	No	primary	FSGS	3	N/A	No	N/A	No	2.6

The table is sorted first by non-genetic / genetic status and then by age of onset.

Legend: FSGS-Focal segmental glomerulosclerosis, MCD-Minimal Change Disease, CNS-Congenital nephrotic syndrome, MHc-Mesangial hypercellularity, DMH-Diffuse mesangial hypercellularity, DMS-Diffuse mesangial sclerosis, MPGN-Membranoproliferative glomerulonephritis, ESRF-End stage renal failure, C1q-C1q nephropathy, FGGS-Focal global glomerulosclerosis, DPGN-Diffuse mesangial proliferative glomerulonephritis, MesPGN - Mesangioproliferative glomerulonephritis, W-White, BA-Black African, BC-Black Caribbean, I-Indian, P-Pakistani, NMO - non mixed origin, As-Asian, Ban-Bangladeshi, CKD – chronic kidney disease, Tx – transplanted, PD-Peritoneal dialysis, HD-Haemodialysis, ** - karyotyping not done, NGS data analysis indicates XY

10.3 Genotypes and phenotypes of patients with likely-pathogenic variants detected by clinical genetic testing (Chapter 4)

Table 10.3: Genotypes and phenotypes of patients with likely-pathogenic variants

Patient number	Sex	Age at onset (years)	Presentation	Ethnicity; FH; Consanguinity	Biopsy	Clinical impact	Pathogenicity	Gene	Nucleotide; segregation	AA	Reference	Mutation prediction: SIFT; PolyPhen	Allele frequency: dbSNP; EVS; ExAC
1	M	6*	SRNS	ND; ND; ND	FSGS	ND	LP	***ACTN4***	c.776C>A	p.(Thr259Asn)	PS	Del; 1.00	NL; NL; NL
2	F	9	Alport	W; Y; N	Alport	ND	LP	***COL4A3***	c.663_664delAG	p.(Arg221Serfs*5)	[404]		
							LP	***COL4A3***	c.3472G>C	p.(Gly1158Arg)	[404]		
3	F	16*	Haematuria; proteinuria, ESRF; hearing loss	ND; Y; ND	ND	ND	LP	***COL4A3***	c.663_664delAG	p.(Arg221Serfs*5)	[404]		
							LP	***COL4A3***	c.1985G>A	p.(Gly662Glu)	PS	Del; 1.00	NL; NL; NL
4	F	25*	SRNS	ND; Y; ND	FSGS	ND	LP	***COL4A3***	c.698G>A; tracks with disease: affected brother	p.(Gly233Glu)	PS	Del; 0.999	NL; NL; NL
5	M	5	Haematuria	ND; ND; ND	Alport	ND	LP	***COL4A3***	c.898G>A	p.(Gly300Arg)	[405]		
							LP	***COL4A3***	c.898G>A	p.(Gly300Arg)	[405]		
6	F	20	Haematuria, proteinuria, TBMN	W; N; N	TBMN	ND	LP	***COL4A3***	c.2083G>A; pat	p.(Gly695Arg)	[406, 407]		

							VUS	***COL4A3***	c.4981C>T; carried by unaffected son	p.(Arg1661Cys)	[408]		
7	M	44*	Alport	ND; ND; ND	ND	ND	LP	***COL4A3***	c.2452G>A; mat; tracks with disease: 4 affected, 2 unaffected	p.(Gly818Arg)	[404]		
							VUS	*COL4A4*	c.4760C>G; mat; tracks with disease: 4 affected, 1 unaffected	p.(Pro1587Arg)	[407]		
8	M	17*	SRNS	S; Y; ND	FSGS	ND	LP	***COL4A4***	c.1598G>A	p.(Gly533Asp)	[404]		
							LP	***COL4A4***	c.1598G>A	p.(Gly533Asp)	[404]		
9	F	78*	Hypertensive nephrosclerosis, ESRF	ND; Y; ND	ND	ND	LP	***COL4A4***	c.2906C>G	p.(Ser969*)	[404]		
10	F	36*	Alport	ND; ND; ND	ND	ND	LP	***COL4A4***	c.3052G>C; mat; tracks with disease: affected mother and mat aunt	p.(Gly1018Arg)	PS	Del; 1.00	NL; NL; 0.00083%
							VUS	*WT1*	c.541C>T; pat unaffected	p.(Pro181Ser) pat	[409]		
							VUS	*WT1*	c.328G>T; pat unaffected	p.(Gly110Trp)	PS	Del, 0.00	NL; NL; NL
11	F	56*	Haematuria, deafness, Alport	W; N; N	ND	ND	LP	***COL4A4***	c.4538G>A	p.(Cys1513Tyr)	[404]		
12	F	25*	Haematuria	W; Y; ND	ND	ND	LP	***COL4A5***	c.367G>A	p.(Gly123Arg)	[410]		
13	M	18	SRNS, bilateral undescended testes, FH severe adult-onset deafness	W; N; N	Not done	ND	LP	***COL4A5***	c.546+1G>T	p.(?)	PS	n/a; n/a	NL; NL; NL
							VUS	*MYH9*	c.2507C>T	p.(Pro83	[411]		

										6Leu)			
14	M	42*	Haematuria, proteinuria, hearing loss	ND; ND; ND	ND	ND	LP	***COL4A5***	c.556G>A	p.(Gly186Ser)	PS	Del; 0.999	NL; NL; NL
15	F	34*	Alport	ND; Y; ND	ND	ND	LP	***COL4A5***	c.1190G>T	p.(Gly397Val)	PS	Del; 1.00	NL; NL; NL
16	F	38*	SRNS	ND; Y; ND	FSGS	ND	LP	***COL4A5***	c.1423G>A; c.4567C>A	p.(Gly475Ser); p.(Pro1523Thr)	[412, 413]		
17	M	9	Alport	BA; Y; N	Alport	ND	LP	***COL4A5***	c.1807G>T	p.(Gly603Cys)	PS	Del; 1.00	NL; NL; NL
18	M	46*	Alport	ND; ND; ND	Alport	ND	LP	***COL4A5***	c.1826G>C; absent in mother (Haematuria detected opportunistically) and unaffected sister	p.(Gly609Ala)	PS	Del; 0.999	NL; NL; NL
19	F	3	Alport	W; N; N	Alport	Imm not started	LP	***COL4A5***	c.1835G>T; neither parent	p.(Gly612Val)	PS. p.(Gly612Asp) [414]	Del; 1.00	NL; NL; NL
20	F	22*	Haematuria, proteinuria, hearing loss	ND; N; ND	Alport	ND	LP	***COL4A5***	c.3270C>G	p.(Tyr1090*)	PS	n/a; n/a	NL; NL; NL
21	M	28	Alport	W; Y; N	FSGS	ND	LP	***COL4A5***	c.3319G>A; mat	p.(Gly1107Arg)	[415]		
22	F	11	SRNS	W; ND; N	FSGS	ND	LP	***COL4A5***	c.4015+2T>C	p.(?)	PS	n/a; n/a	NL; NL; NL
23	F	15	Haematuria, proteinuria	Brazilian; Y; N	Not done	Avoid biopsy	LP	***COL4A5***	c.4415_4416delinsCT	p.(Arg1472Pro)	PS	Del; 0.999	NL; NL; NL
24	M	0.5	Haematuria, hearing loss	ND; Y; ND	Alport	ND	LP	***COL4A5***	c.4480delT; mat	p.(Ser1494Leufs*60)	PS	n/a; n/a	NL; NL; NL
25	M	38*	CMT + NS	ND; Y; ND	FSGS	ND	LP	***INF2***	c.148T>G; tracks with disease: 5 affected, 1 unaffected	p.(Tyr50Asp)	PS	Del; 0.999	NL; NL; NL
							VUS	*COL4A4*	c.778G>A; does not track	p.(Val260Ile)	PS	Del; 0.007	NL; NL; 0.0042%

									with disease: 5 affected				
26	M	24	SRNS	W; N; N	FSGS	Imm not started	LP	***INF2***	c.494T>G; detected in affected son; absent in unaffected son and father	p.(Leu165Arg)	PS. p.(Leu165Pro) [293]	Del; 0.999	NL; NL; NL
27	F	25	SRNS, CKD	W; N; N	FSGS	No	LP	***INF2***	c.640C>T; pat; detected in affected father	p.(Arg214Cys)	[61, 293, 416]		
28	M	0.1	CNS, Pierson syndrome	ND; ND; ND	Not done	Palliative care	LP	***LAMB2***	c.825T>A; mat	p.(Tyr275*)	[417]		
							LP	***LAMB2***	c.825T>A; pat	p.(Tyr275*)	[417]		
29	M	0	CNS	W; N; N	ND	Antenatal testing in subsequent pregnancy	LP	***LAMB2***	c.1477delT; mat	p.(Cys493Alafs*4)	[417, 418]		
							LP	***LAMB2***	c.3523delC; pat	p.(Gln1175Serfs*37)	PS	n/a; n/a	NL; NL; NL
30	M	0	CNS, died at 1 week	Ir; Y; Y	Not done	ND	LP	***LAMB2***	c.1814delG	p.(Gly605Valfs*23)	PS	n/a; n/a	NL; NL; NL
							LP	***LAMB2***	c.1814delG	p.(Gly605Valfs*23)	PS	n/a; n/a	NL; NL; NL
31	F	18	SRNS	Pa; N; N	Tubulo-interstitial disease	ND	LP	***LMX1B***	c.668G>A; neither parent	p.(Arg223Gln)	[419]		
32	F	17	SRNS	W; N; N	FSGS	Imm not started	LP	***LMX1B***	c.737G>A; absent in unaffected father and brother	p.(Arg246Gln)	[67, 420]		
33	F	14	SRNS	W; Y; N	FSGS	ND	LP	***LMX1B***	c.737G>A	p.(Arg246Gln)	[67, 420]		
34	M	2	SRNS	ME; N; N	FSGS	Cessation of Imm	LP	***LMX1B***	c.737G>A	p.(Arg246Gln)	[67, 420]		
35	F	13*	SRNS, thrombocytope	ND; ND; ND	ND	ND	LP	***MYH9***	c.287C>T	p.(Ser96Leu)	[421, 422]		

			nia										
36	F	10*	NS, thrombocytopenia, FH of same	ND; Y; ND	ND	ND	LP	***MYH9***	c.2152C>T	p.(Arg718Trp)	[423, 424]		
37	M	0	SRNS, hypomagnesaemia with secondary hypocalcaemia, focal seizures, hypothyroidism, Arnold-Chiari malformation	In; N; N	Finnish type	ND	LP	***NPHS1***	c.58+1G>T; c.2600G>A; mat	p.(?)	[74]		
							LP	***NPHS1***	c.320C>T; pat	p.(Ala107Val)	[74]		
38	M	0.1	CNS, hypomagnesaemic seizures	Ban; N; Y	Not done	Imm not started	LP	***NPHS1***	c.320C>T; mat	p.(Ala107Val)	[74]		
							LP	***NPHS1***	c.320C>T; pat	p.(Ala107Val)	[74]		
39	F	0	CNS, IUGR	ND; N; ND	ND	ND	LP	***NPHS1***	c.325T>C; mat	p.(Tyr109His)	PS	Del; 0.993	NL; NL; 0.00087%
							LP	***NPHS1***	c.1868G>T; pat	p.(Cys623Phe)	[425]		
40	M	0.1	CNS, psychomotor delay	W; N; N	Not done	Imm not started	LP	***NPHS1***	c.325T>C; pat	p.(Tyr109His)	PS	Del; 0.993	NL; NL; 0.00087%
							LP	***NPHS1***	c.3442C>T; mat	p.(Gln1148*)	[426]		
41	M	0	CNS, deafness	W; N; N	FSGS	ND	LP	***NPHS1***	c.532C>T; mat	p.(Gln178*)	[71]		
							LP	***NPHS1***	c.1243dupC; pat	p.(Leu415Profs*4)	PS	n/a; n/a	NL; NL; NL
42	M	0	CNS, placentomegaly and hepatosplenomegaly and single palmar creases	Filipino; N; ND	ND	ND	LP	***NPHS1***	c.565G>T	p.(Glu189*)	[427]		
							LP	***NPHS1***	c.1379G>A	p.(Arg46	[71]		

										0Gln)				
43	F	0	CNS	W; N; N	Not done	ND	LP	***NPHS1***	c.736G>T	p.(Glu246*)	[428]			
							LP	***NPHS1***	c.1868G>T	p.(Cys623Phe)	[425]			
44	F	0	CNS	W; N; N	Not done	ND	LP	***NPHS1***	c.866G>A; pat	p.(Trp289*)	[74]			
							LP	***NPHS1***	Exon 23-29del; mat	p.(?)	PS	n/a; n/a	NL; NL; NL	
45	F	0.4*	CNS	ND; ND; ND	DMS	ND	LP	***NPHS1***	c.1235delG	p.(Gly412Valfs*2)	PS	n/a; n/a	NL; NL; NL	
							LP	***NPHS1***	c.3481+1G>T	p.(?)	[74]			
46	M	2*	SRNS	ND; ND; ND	ND	ND	LP	***NPHS1***	c.1868G>T	p.(Cys623Phe)	[425]			
							LP	***NPHS1***	c.2335-1G>A	p.(?)	[283]			
47	M	0	CNS	W; N; N	Other	Assess risk of recurrent FSGS after transplant	LP	***NPHS1***	c.2227C>T; pat	p.(Arg743Cys)	[70, 283, 429]			
							LP	*NPHS1*	c.2309C>T; c.2335-1G>A both mat	p.(Pro770Leu); p.(?)	PS; [283]	Del; 0.824	rs115976159; NL; 0.0099%	
48	F	0.1	CNS	W; N; N	Finnish type	Imm not started	LP	***NPHS1***	c.2335-1G>A; mat	p.(?)	[283]			
							LP	***NPHS1***	c.2335-1G>A; pat	p.(?)	[283]			
49	F	11*	SRNS	W; Y; N	ND	ND	LP	***NPHS2***	c.413G>A	p.(Arg138Gln)	[296]			
							LP	***NPHS2***	c.378+1_378+2delinsTG	p.(?)	PS	n/a; n/a	NL; NL; NL	
50	M	13	SRNS	W; N; N	FSGS	ND	LP	***NPHS2***	c.413G>A	p.(Arg138Gln)	[296]			
							LP	***NPHS2***	c.868G>A	p.(Val290Met)	[430]			

51	M	0.1	CNS, ESRF, died at 2y	In; N; N	FSGS	Genetic testing done post-mortem	LP	***NPHS2***	c.419G>A; mat	p.(Gly140Glu)	PS	Del; 1.00	NL; NL; 0.00082%
							LP	***NPHS2***	c.419G>A; pat	p.(Gly140Glu)	PS	Del; 1.00	NL; NL; 0.00082%
52	F	3.5	SRNS	Mix Afg/In; Y; Y	FSGS	Cessation of Imm	LP	***NPHS2***	c.562G>T; mat	p.(Glu188*)	[431]		
							LP	***NPHS2***	c.562G>T; pat	p.(Glu188*)	[431]		
53	M	12*	SRNS	ND; ND; ND	ND	ND	LP	***NPHS2***	c.871C>T	p.(Arg291Trp)	[432]		
							LP	*NPHS2*	c.686G>A	p.(Arg229Gln)	[69, 297]		
54	F	11	SRNS	W; N; N	FSGS	Imm not started	LP	***NPHS2***	c.890C>T; pat	p.(Ala297Val)	[286, 299]		
							LP	***NPHS2***	c.686G>A; mat	p.(Arg229Gln)	[69, 297]		
							VUS	*COL4A4*	c.232C>T	p.(Pro78Ser)	PS	Del; 0.008	NL; NL; 0.00083%
55	M	0.9*	CNS, severe pulmonary valve stenosis	W; N; N	ND	ND	LP	***NPHS2***	c.1032delT; pat	p.(Phe344Leufs*4)	[286]		
							LP	***NPHS2***	Exon 2 del; mat	p.(?)	PS	n/a; n/a	NL; NL; NL
56	M	2.5	SRNS	K; Y; Y	FSGS	Imm not continued. Assess risk of recurrent FSGS after transplant	LP	***PLCe1***	c.1477C>T; mat	p.(Arg493*)	[60, 303]		
							LP	***PLCe1***	c.1477C>T; pat	p.(Arg493*)	[60, 303]		
							VUS	*APOL1*	c.558delA; pat	p.(Gly187Alafs*19)	PS	n/a; n/a	NL; NL; 0.0041%
57	F	2.8	SRNS	Afg; N; N	FSGS	ND	LP	***PLCe1***	c.1477C>T	p.(Arg493*)	[60, 303]		
							LP	***PLCe1***	c.1477C>T	p.(Arg493*)	[60, 303]		
							VUS	*CD2AP*	c.1511G>A	p.(Arg504His)	PS	Tol; 0.952	NL; NL; 0.00249%
58	F	22	SRNS, tremor	W; N; N	FSGS	Planned	LP	***SCARB2***	c.434_435dup	p.(Trp14	[133, 433]		

			& ataxia, suspected AMRF			Imm not started until genetic test known				6Serfs*16)			
							LP	***SCARB2***	c.704+5G>A	p.(?)	[434]		
59	M	7	SRNS, short stature, mild central hypoventilation	W; N; N	FSGS	ND	LP	***SMARCAL1***	c.415_416delTT	p.(Leu139Glufs*3)	PS	n/a; n/a	NL; 0.01%; 0.0016%
							LP	***SMARCAL1***	c.2114C>T	p.(Thr705Ile)	[91]		
							VUS	*ALMS1*	c.11449C>T	p.(Gln3817*)	[435]		
							Carrier	*PMM2*	c.422G>A	p.(Arg141His)	[436]		
60	M	39	SRNS, unilateral blindness	W; ND; N	FSGS	ND	LP	***TRPC6***	c.2690A>C	p.(Glu897Ala)	PS. p.(Glu897Lys) [437]	Del; 0.995	NL; NL; NL
61	M	37*	SRNS	W; Y; ND	FSGS	ND	LP	***WT1***	c.1016A>G; tracks with disease: 6 affected (tested elsewhere, personal communication)	p.(His339Arg)	PS	Del; 0.991	NL; NL; NL
62	M	11	SRNS, undescended testes	W; N; N	FSGS	Imm not started	LP	*WT1*	c.1091T>G	p.(Phe364Cys)	PS	Del; 0.89	NL; NL; NL
63	F	2	SRNS, seizures at 6m	W; N; ND	Not done	ND	LP	***WT1***	c.1097G>A	p.(Arg366His)	[65]		
64	M	3*	SRNS, undescended testes, penile anomaly	ND; ND; ND	ND	ND	LP	***WT1***	c.1133C>T; absent in both unaffected parents	p.(Thr378Ile)	[81]		
							LP	***COL4A3***	c.2452G>A; pat unaffected	p.(Gly818Arg)	[404]		
65	F	30	Nephrotic in pregnancy	W; Y; N	FSGS	No planned Imm	LP	***WT1***	c.1169G>A; pat affected	p.(Arg390Gln)	PS. p.(Arg390*) [80]	Del; 0.999	NL; NL; NL

							VUS	*COL4A4*	c.4334-3C>T; pat	p.(?)	PS	n/a; n/a	NL; NL; NL
66	F	0.6	Proteinuria, ESRF	ND; ND; ND	Multi-segmental sclerosing lesions	Already in ESRF at presentation	LP	***WT1***	c.1180C>T; absent in both unaffected parents	p.(Arg394Trp)	[65, 438]		
							VUS	*LAMB2*	c.4331G>A; mat	p.(Gly1444Glu)	PS	Del; 0.99	NL; NL; NL
67	F	1	SRNS	ND; ND; ND	ND	ND	LP	***WT1***	c.1180C>T	p.(Arg394Trp)	[65, 438]		
68	M	0.3*	CNS	ND; ND; ND	ND	ND	LP	***WT1***	c.1181G>A; absent in both unaffected parents	p.(Arg394Gln)	[439, 440]		
69	M	3.3	SRNS, ADHD	M; N; Y	DMS	Imm not started	LP	***WT1***	c.1228+5G>A; absent in both unaffected parents	p.(?)	[81, 441]		
70	F	3	SRNS	W; N; N	FSGS	ND	LP	***WT1***	c.1228+5G>A; absent in both unaffected parents	p.(?)	[81, 441]		
71	F	2.5	SRNS	W; N; N	FSGS	ND	LP	***WT1***	c.1228+5G>A; absent in both unaffected parents	p.(?)	[81, 441]		

* Denotes age at genetic testing where age at disease onset was not available.
Genes in bold type are the main causative gene in that patient.

Legend:

AA, amino acid; ADHD, attention-deficit hyperactivity disorder; Afg, Afghanistani; AMRF, action myoclonus renal failure syndrome; BA, Black African; Ban, Bangladeshi; CKD, chronic kidney disease; CMT, Charcot Marie Tooth disease; CNS, congenital nephrotic syndrome; Del, deleterious; DMS, diffuse mesangial sclerosis; ESRF, end stage renal failure; FH, family history; FSGS, focal segmental glomerulosclerosis; Imm, immunosuppression; In, Indian; Ir, Iranian; IUGR, intra-uterine growth restriction; K, Kurdish; m, months; LP, likely-pathogenic; M, Moroccan; mat, maternal; MCD, minimal change disease; ME, Middle Eastern; N, no; n/a, not available; ND, not done/no data; NL, not listed; NNP, non-neutral polymorphism; NS, nephrotic syndrome; Pa, Pakistani; pat, paternal; PS, present study; S, Slovakian; SRNS, steroid resistant nephrotic syndrome; TBMN, thin basement membrane nephropathy; Tol, tolerated; VUS, variant of unknown significance; W, White; y , years; Y, yes

10.4 Publications resulting from this thesis

1. Bierzynska A, McCarthy HJ, Soderquest K, Sen ES, Colby E, Ding WY, et al. Genomic and clinical profiling of a national nephrotic syndrome cohort advocates a precision medicine approach to disease management. Kidney international. 2017;91(4):937-47.
2. Sen ES, Dean P, Yarram-Smith L, Bierzynska A, Woodward G, Buxton C, et al. Clinical genetic testing using a custom-designed steroid-resistant nephrotic syndrome gene panel: analysis and recommendations. J Med Genet. 2017;54(12):795-804.